FORMATION AND CONTROL OF BIOFILMS OF THERMO-RESISTANT STREPTOCOCCI ON STAINLESS STEEL

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ABSTRACT

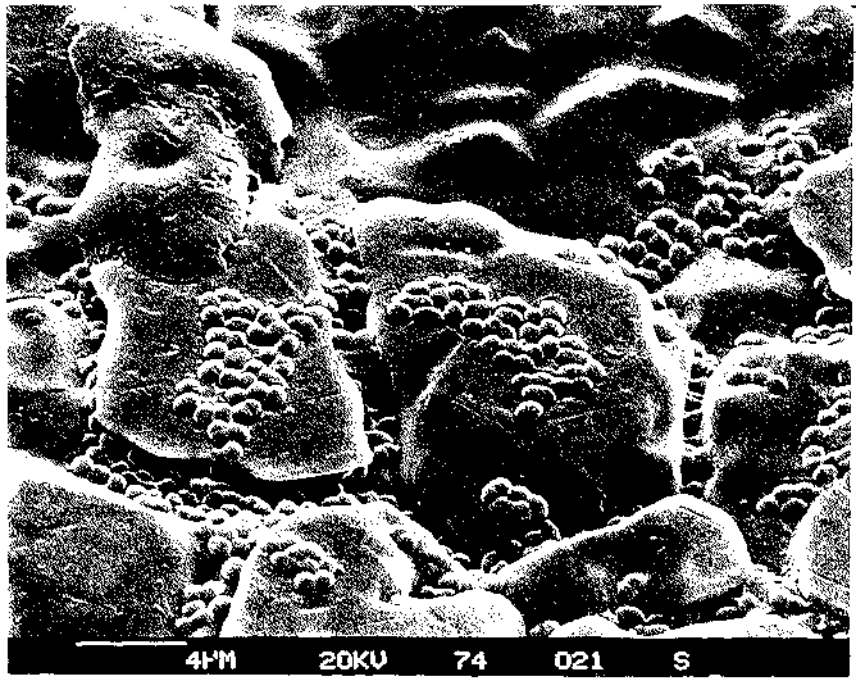
The aim of this study was to develop improved methods of controlling biofilms of thermo-resistant streptococci in dairy manufacturing plant.

A method to rapidly and accurately detect viable cells of thermo-resistant streptococci on stainless steel surfaces involving the use of the Malthus microbiological growth analyser was developed. A modified Robbins device was designed and installed in a dairy manufacturing plant to monitor biofilm development and obtain isolates for study. These studies confirmed that routine cleaning programmes were not eliminating biofilms of thermo-resistant streptococci from the stainless steel surface. The isolates obtained were identified using biochemical and molecular techniques. As well as the expected *Streptococcus thermophilus,* a new species, *S. waiu* representing 24% of the isolates was also described. Molecular techniques (polymerase chain reaction and fluorescent *in situ* hybridisation) were developed to rapidly identify the bacteria. The cell surface hydrophobicity of all isolates was determined, with those obtained from dairy manufacture being highly hydrophobic compared with mixed hydrophobicity in the general population.

There was no correlation between many factors often associated with adhesion (such as hydrophobicity, polysaccharide production, surface charge) and the rate of cell adhesion. However, treatment of the bacteria with proteolytic agents reduced the number of all isolates adhering to stainless steel by approximately 100-fold. A 55 kDa protein with an N-terminal sequence matching that of β-lactoglobulin was identified as being associated with adhesion, through comparisons between cell proteins separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis

before and after treatment with proteolytic agents. Further evidence of the involvement of this protein in adhesion was the reduction in adhesion following treatment of the cells with specific antiserum to the 55 kDa "adhesion protein". The presence of the protein on the surface of the cells was demonstrated by immunolabelling.

A continuous flow laboratory reactor was developed to generate biofilms of thermo-resistant streptococci on stainless steel surfaces in the presence of skim milk. Trials using biofilms developed in laboratory reactors and on the surface of coupons in pilot plants, indicated that chemicals routinely used in dairy manufacturing plants were inadequate to remove or inactivate thermo-resisant streptococci. Proteolytic enzyme treatments removed more bacteria from the surface than any other treatment, reducing the total number of cells by at least 100-fold. This was confirmed in a pilot-scale trial using a commercial proteolytic-enzyme-based cleaner. In addition, no viable cells were detected following treatment with this cleaner. Proteolytic enzyme cleaners may be more effective than the caustic and acid cleaners for the routine cleaning of biofilms of thermo-resistant streptococci from dairy manufacturing plants.



Frontispiece Scanning electron micrograph of *Streptococcus thermophilus* colonising 316 stainless steel with a 2b surface finish as used in dairy manufacturing plants. The cells are in clumps rather than as individual cells and appear to be preferentially colonising the interfaces between the oxide grain boundaries on the surface of this stainless steel sample.
Magnification = 3600 x.

LIST OF PUBLICATIONS

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3. Flint, S. H., Brooks, J. D., Bremer, P. J. (1996). Factors affecting the attachment of *Streptococcus bovis* and *Bacillus cereus* to stainless steel surfaces. *New Zealand Microbiological Society Conference,* Christchurch, October 1996.

4. Flint, S. H., Brooks, J. D., Bremer, P. J. (1996). The influence of cell surface properties of thermophilic streptococci on attachment to stainless steel. *American Society for Microbiology Conference on Microbial Biofilms*, Utah, USA, October 1996.

5. Flint, S. H., Brooks, J. D., van den Elzen, H., Bremer, P. J. (1997). Biofilms in dairy manufacturing plant - a threat to product quality. *New Zealand Institute of Food Science and Technology Conference,* Napier, July 1997.

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6. Flint, S. H., Brooks, J. D., Bremer, P. J. (1997). The influence of cell surface properties of thermophilic streptococci on attachment to stainless steel. *New Zealand Institute of Food Science and Technology Conference,* Napier, July 1997.

7. Flint, S. H., Bremer, P. J., Brooks, J. D. (1997). The influence of topography on the development of biofilms of thermophilic streptococci on stainless steel surfaces. *New Zealand Institute of Food Science and Technology Conference,* Napier, July 1997.

8. Flint, S. H., Ward, L. J. H., Brooks, J. D., Bremer, P. J. (1997). Description of *Streptococcus waiu* sp. nov. *New Zealand Microbiological Society Conference,* Rotorua, November 1997.

9. Flint, S. H., Brooks, J. D., Bremer, P. J. (1997). Adhesion mechanisms of thermophilic streptococci. *New Zealand Microbiological Society Conference,* Rotorua, November 1997.

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Chapter 1

INTRODUCTION

This thesis is part of a programme of research, centred at Massey University, Palmerston North, New Zealand, studying the development and control of biofilms in dairy manufacturing plant. Biofilm formation has been studied in a wide range of environments for many years. Biofilms may be useful as in the case of waste water treatment and acetic acid production. However, biofilms are more often cited for potential problems they may present including contamination of water distribution systems, dental caries and contamination of medical prostheses.

The mechanism by which biofilms develop is not completely understood. There are several proposed models for the development of biofilms. These can be simplified into three main stages - adhesion, growth and detachment. Adhesion involves a complex interaction between a substrate and the microorganism and is influenced by the surrounding environment. Biofilm growth is often associated with the production of extracellular polysaccharide material that may bond the biofilm colony together, forming a more secure attachment to the substrate and protecting the bacteria within the biofilm from external factors that would otherwise damage the bacteria. Detachment enables the bacteria to spread and colonise other regions of the substrate and also results in the contamination of the environment.

Although biofilms have been studied in many environments. It is only since the early 1980's that biofilms were recognised as a concern in food processing environments. Bacterial adherence to meat was the first food-related biofilm problem to be recognised with the potential for spoilage and pathogen contamination. Food contact surfaces were later found to be prone to microbial colonisation from a wide variety of microorganisms. In a mature biofilm these

microorganisms may be difficult to remove and have been shown to be more resistant to common cleaning and sanitising treatments.

The shedding of bacteria from biofilms developing in dairy manufacturing plant is now recognised as a major cause of microbial contamination of dairy products. Biofilm colonisation in manufacturing plants potentially affects all manufactured products. There are many sites in a dairy manufacturing plant where biofilms are likely to develop. The nature of the biofilm is likely to depend on the environmental conditions at specific parts of the dairy manufacturing process. The development of biofilms in pasteurisers is the most widely reported, with the potential to cause contamination of all dairy products. The development of biofilms of thermo-resistant (also referred to as thermophilic) streptococci in pasteurisers has been monitored in industrial and pilot-scale plant. However, these biofilms are poorly understood and difficult to control, with manufacturing plants relying on more frequent cleaning to prevent microbial contamination reaching unacceptable levels. The microbial composition of these biofilms is not completely understood, the adhesion mechanism has not been studied and there are no reports exploring the effectiveness of current and alternative cleaning methods.

This thesis results from the New Zealand dairy industry's recognition of potential problems in maintaining product quality that may result from biofilms in dairy manufacturing plants. The focus of this study was to define the microbial composition of biofilms of thermo-resistant streptococci developing in the cooling section of milk pasteurisers, develop a fundamental understanding of the mechanisms of adhesion of these bacteria and examine potential methods to improve the control of these biofilms. This study aimed to develop a greater awareness of biofilm development in the dairy environment and to develop and apply techniques to enable further studies of biofilms of other bacterial species at different sites in dairy manufacturing plant.

Chapter 2

BIOFILMS IN DAIRY MANUFACTURING PLANT - DESCRIPTION, CURRENT CONCERNS AND METHODS OF CONTROL. A REVIEW OF THE LITERATURE

2.1 INTRODUCTION

The dairy industry manufactures a wide range of perishable (*e.g.* butter and cheese) and semi perishable (milk powder and casein) foods. Strict adherence to microbiological guidelines is essential to maintain the stability, flavour and functionality of these products. Milk is produced by the cow as a sterile secretion, However, contamination can occur during milking, transportation, storage and processing. During milking, contamination frequently occurs from microorganisms present on the teats and udder or from the milking machine. Milk stored on the farm (for up to 48 h) is maintained at 7°C, limiting the growth of non-psychrotrophic microorganisms but allowing some growth of psychrotrophic microorganisms (*e.g.* *Pseudomonas* species) which may be present. During subsequent transportation and storage on the manufacturing site, there is potential for contamination from the transfer lines and storage vessels. The milk at this stage of the manufacturing process in New Zealand is not refrigerated, the processor relying on the temperature of the bulk milk to limit growth of microorganisms until the start of manufacture.

The manufacturing process relies on pasteurisation (72°C for 15 s) at an early stage of manufacture to destroy heat-sensitive spoilage and pathogenic bacteria present in the raw milk. Further processing steps (pH reduction through the addition of starter, drying, the addition of salt and cooling) are designed to limit the growth of the thermoduric bacteria that survive this heat treatment. At all stages in processing, good hygiene of the manufacturing plant is essential to ensure that the manufacturing plant does not contaminate the product stream.

Biofilms present on the surface of milk processing equipment are increasingly implicated as a significant source of contamination of dairy products (Bouman *et al.*, 1981; Koutzayiotis, 1992; Limsowtin and Powell, 1996). Contamination is currently receiving increased interest due to the trend towards producing products with longer shelf life, stricter hygiene requirements, increasing awareness of the problems posed by pathogens such as *Listeria monocytogenes*, the trend toward longer processing

runs (Sutherland, 1995), automation and the increasing complexity of equipment (Notermans, 1994).

This review describes the unique features of biofilms that develop in dairy manufacturing plants and the problems associated with their development, and discusses the parameters that influence the nature and rate of biofilm development. Methods used to detect the presence of biofilms as well as current methods to control biofilm development are described. The potential of other cleaning regimes to control biofilms and the requirement for further research in this field are discussed.

2.2 BIOFILMS IN DAIRY MANUFACTURING PLANTS

A biofilm is an aggregation of microbial cells and their associated extracellular polymeric substances or polysaccharides (EPS), actively attached to, growing on and multiplying on a surface. Biofilms can be divided into two categories: (1) biofilms termed "process" biofilms, *viz.* those that form on surfaces (*e.g.* heat exchanger) in direct contact with flowing product; (2) biofilms that form in the general food processing environment. As biofilm formation and the consequences in the general food processing environment have recently been reviewed (Mattila-Sandholm and Wirtanen, 1992; Carpentier and Cerf, 1993; Notermans, 1994; Zottola and Sasahara, 1994; Bower *et al.*, 1996), this review will focus mainly on describing the characteristics and significance of process biofilms in dairy manufacturing plants. The development of biofilms of *Listeria monocytogenes* in the processing environment will also be included due to their importance to the dairy industry.

2.2.1 Characteristics of process biofilms in dairy manufacturing plants

Process biofilms in dairy manufacturing plants have a number of characteristics that distinguish them from biofilms usually associated with food processing facilities. In process biofilms, a single species often predominates (Hup and Stadhouders, 1979; Bouman *et al.*, 1982). This may be a result of the heat treatment of milk

(pasteurisation) which reduces the competition from heat-sensitive Gram-negative species and allows the thermoresistant species that are often found in milk (*e.g. Streptococcus thermophilus*) to grow. Process biofilms are also characterised by their rapid development with numbers of bacteria up to 6.0 $\log_{10}$ cells cm^{-2} being recorded in the regeneration section of a pasteuriser after 12 h of operation (Bouman *et al.*, 1982) (Figures 2.1 and 2.2).

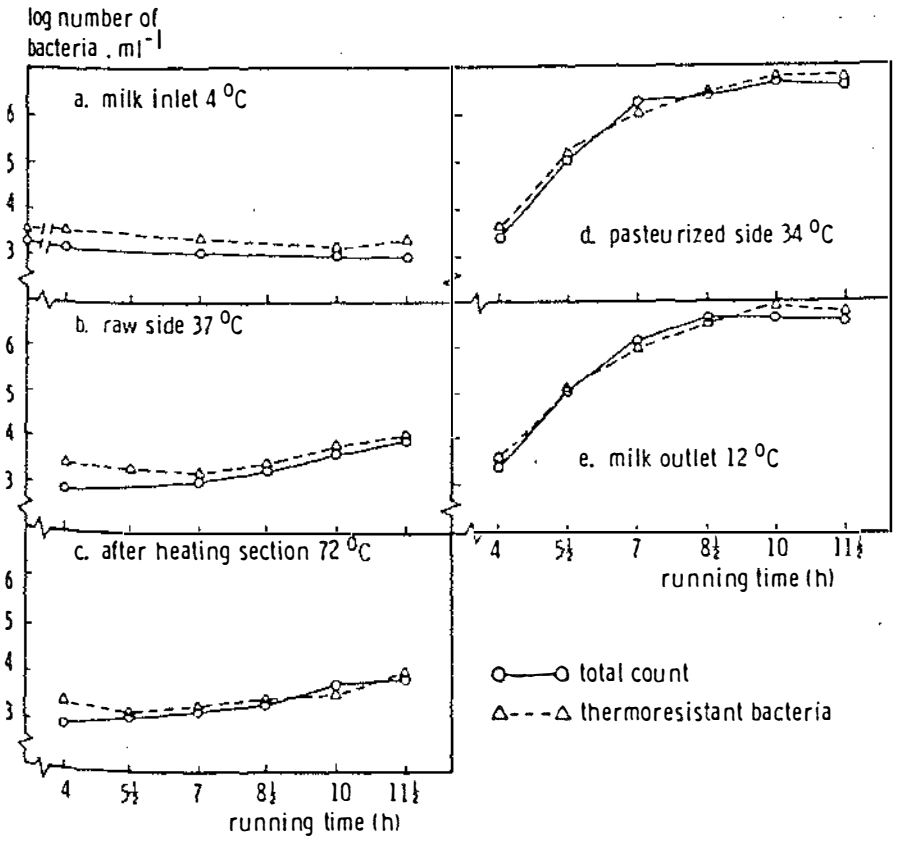


Figure 2.1 Total number of bacteria and thermo-resistant bacteria in the milk during pasteurisation - samples taken at different points during the process. (From Bouman *et al.*, 1982; reprinted with permission from *Journal of Food Protection*. Copyright held by the International Association of Milk, Food and Environmental Sanitarians, Inc.)

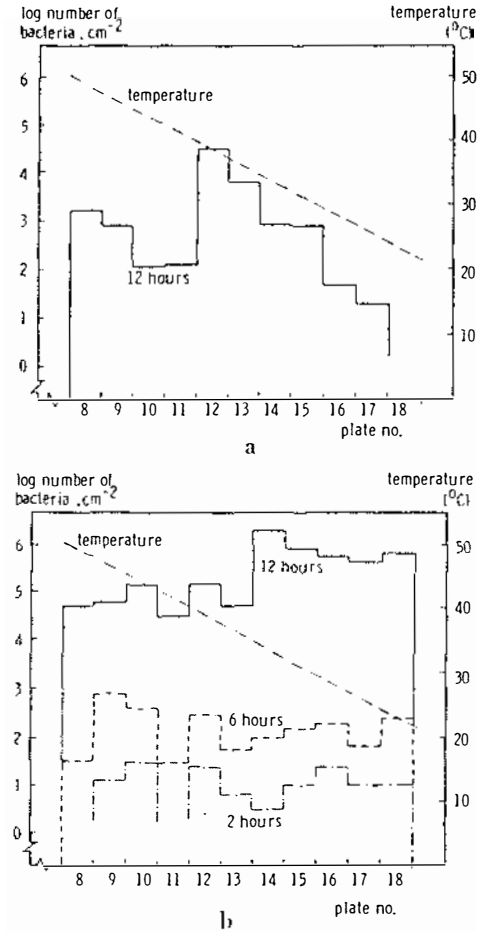


Figure 2.2 Bacteria and temperature of the walls of the regeneration section of a heat exchanger: (a) raw side, (b) pasteurised side -"hours" refers to the length of time the pasteuriser has been operating. (From Bouman *et al.*, 1982; reprinted with permission from *Journal of Food Protection*. Copyright held by the International Association of Milk, Food and Environmental Sanitarians, Inc.).

A biofilm will develop on any surface exposed to a non-toxic aqueous environment. The rate and extent of biofilm accumulation is governed by a number of processes, including adsorption and growth of bacterial cells (Escher and Characklis, 1990). In the following section, these steps in process biofilm development will be described.

In the adhesion phase, the development of a protein film on the substrate has been postulated to be an essential prerequisite for the formation of a biofilm in a dairy processing environment (Kirtley and McGuire, 1989). In other studies, phospholipid deposition has been reported to precede the adhesion and growth of *Pseudomonas* species on the surface of milk processing lines (Maxcy, 1972).

Austin and Bergeron (1995) and others have reported the ability of bacteria to adhere to rubber gaskets and stainless steel surfaces. Factors affecting adhesion include the growth phase and viability of the bacteria, the length of time the bacteria are in contact with the surface, the temperature of the suspending medium and surface, the surface roughness and the concentration of milk soil on surfaces, the flow rate and cell surface properties, such as hydrophobicity and surface charge (Langeveld *et al.*, 1972; Hup *et al.*, 1979; Bouman *et al.*, 1982; Hoffman, 1983; Speers and Gilmour, 1985; Czechowski, 1990; van der Mei *et al.*, 1993).

The relationship between surface topography and the adhesion of bacteria has been studied intermittently over the last 40 years (Masurovsky and Jordan, 1958; Langeveld *et al.*, 1972; Hoffman, 1983; Mafu *et al.*, 1990; Pedersen, 1990; Leclercq-Perlat and Lalande, 1994; Wirtanen *et al.*, 1995). The effect of surface topography on bacterial adhesion is taken seriously by regulatory authorities. Regulations for the food processing industry (particularly the dairy industry) specify that surfaces in contact with food products, must conform to US 3-A-Sanitary Standard 01-07 (1990), which requires a finish of a No. 4 grade which is usually achieved through precision grinding using silicon carbide 150 grid (mesh $inch^{-1}$). In addition, dairy standard DIN 11480-1978 (1978), requires that surface roughness does not exceed values (R_a) of

0.8 μm.

However, opinions vary as to the effects of surface roughness on bacterial adhesion and growth. Some researchers have reported that there is a positive correlation between adhesion and increased surface roughness (Masurovsky and Jordan, 1958; Hoffman, 1983; Pedersen, 1990; Leclercq-Perlat and Lalande, 1994; Wirtanen *et al.*, 1995), whereas other researchers have reported no correlation between surface irregularities or roughness and the ability of bacteria to attach (Langeveld *et al.*, 1972; Mafu *et al.*, 1990; Vanhaecke *et al.*, 1990). The apparent conflict in these two opposing observations is probably related to the degree of surface roughness studied, the bacterial species, the physico-chemical parameters of the surface, the bulk fluid phase under study and the method used to detect bacteria on the surface.

Adhesion of bacteria may be increased or reduced in dairy fluids depending on the composition of the dairy fluid and the type of bacteria. Adhesion has been reported to be reduced in the presence of whole milk but increased by the presence of lactose and non-casein protein solutions (Speers and Gilmour, 1985; Suárez *et al.*, 1992; Criado *et al.*, 1994). An association of the bacteria with milk fat globules and the effect of natural antibodies have been suggested as possible reasons for this reduction of bacterial adhesion. However, specific milk proteins, in particular bovine serum albumin, inhibit the adhesion of *L. monocytogenes* (Al-Makhlafi *et al.*, 1995). This is in line with observations in the medical field where albumin is recognised as a protein preventing bacterial adhesion (Bower *et al.*, 1996).

The origin, age and temperature of the incoming milk have been postulated to alter the rate at which populations of thermo-resistant, thermophilic streptococci develop in cheese milk pasteurisers (Hup *et al.*, 1979). The growth of thermo-resistant streptococci in a biofilm has been reported to be slower in raw milk compared with pasteurised milk, although adhesion is the same in both raw and pasteurised milk (Driessen *et al.*, 1984). This suggests that there are growth inhibitory factors in raw milk that are destroyed by heat treatment. Further studies on the importance of

different components of milk and other dairy fluids in the formation of biofilms in dairy manufacturing plant are required.

The type of bacteria in milk samples may reflect the growth of biofilms. For example, the greater numbers of thermo-resistant streptococci and *Bacillus* species in pasteurised milk compared with raw milk (Robinson, 1990) may be explained by contamination from the detachment of organisms from biofilms.

Studies of the characteristics of bacteria forming biofilms on the surface of dairy manufacturing plant are providing information to assist our understanding of how these biofilms develop and may lead to improved methods of control. Thermo-resistant streptococci isolated from dairy processing plant pasteurisers were all found to be hydrophilic and slightly negatively charged (van der Mei *et al.*, 1993). Adhesion of the Gram-negative rod *Pseudomonas fluorescens* occurred most readily with exponential and stationary phase cells, with greater numbers attaching at 25°C compared with 10°C (Czechowski, 1990). Inactivated cells killed by formaldehyde or heat (60°C for 30 min) reduced the numbers of cells adhering by 65-80% and 95-99% respectively. Adhesion took less than 1 min and greater numbers attached to stainless steel in dilute milk than in standard milk.

Many studies have examined the factors involved in the development of biofilms containing *Listeria* species in meat and dairy processing environments where this organism poses a threat to the safety of the products produced (Blackman and Frank, 1996; van der Veen-Koers, 1997). *L. monocytogenes* will attach to stainless steel surfaces (Herald and Zottola, 1987) and increased resistance to sanitisers has been reported when this pathogen develops as a biofilm (Frank and Koffi, 1990; Lee and Frank, 1991; Oh and Marshall, 1995). In a biofilm, *L. monocytogenes* is associated with various other bacteria including staphylococci and lactobacilli (Frank and Koffi, 1990).

In studies by Jeong and Frank (1994a and 1994b), the extent of initial adhesion of

L. monocytogenes varied considerably depending on the strains of competitive bacteria, as did the ability to replicate in biofilms in meat and dairy processing plants. Reduced adhesion occurred in the presence of selected *Pseudomonas, Bacillus* and *Streptococcus* species. The presence of *Staphylococcus, Flavobacterium, Corynebacterium, Micrococcus* and another *Pseudomonas* species had no effect on adhesion. None of the cultures tested enhanced the adhesion of *L. monocytogenes* and none of the competing isolates tested eliminated *Listeria* from the biofilm. In contrast, Sasahara and Zottola (1993) reported results from experiments with biofilms of *Pseudomonas fragi*, showing that established biofilms offered an environment that encouraged the establishment of populations of pathogenic bacteria such as *L. monocytogenes* within the processing facility.

The adhesion of *Listeria* and the development of a biofilm appear to be a result of competition for adhesion sites and the function of competitive influences of other microflora in the biofilm, with the fastest growing species within a biofilm often dominating (Banks and Bryers, 1991; Jeong and Frank, 1994a and 1994b).

2.2.2 Concerns associated with biofilms in dairy manufacturing plants

Biofilms are of concern in dairy manufacturing plants, as bacteria within biofilms are more difficult to eliminate than free living cells (Wirtanen and Mattila-Sandholm, 1992a; Mattila-Sandholm and Wirtanen, 1992; Mosteller and Bishop, 1993) and once established can act as a source of contamination of product and other surfaces.

Microbial contamination is a major cause of poor quality dairy products. A summary of the predominant biofilm microflora in different dairy processes is given in Table 2.1. The following sections present examples of biofilm induced problems that have been reported in different dairy manufacturing operations.

2.2.2.1 Cheese manufacture

The development of biofilms consisting of thermo-resistant streptococci in pasteurisers and thermalisers has caused contamination of cheese milk resulting in associated problems with cheese quality. In one manufacturing plant, the levels of thermo-resistant streptococci in milk from the pasteuriser reached over 6.0 $\log_{10}$ cells ml^{-1} after 7-8 h of operation. Gouda cheese produced from this milk had an unsuitable texture and an unclean yeasty flavour (Hup *et al.,* 1979). The length of time the milk was in the pasteuriser was too short for the increase in numbers to be due to bacterial growth and therefore the presence of a contaminating biofilm was postulated (Driessen and Bouman, 1979). The adhesion of bacteria and protein to the plates of pasteurisers was observed to occur in the temperature range 30-50°C (Hup *et al.,* 1979) with colonisation reported to be localised in the regeneration section of pasteurisers (Lehmann *et al.,* 1990). Bacteria associated with the cooling section of a model pasteuriser were found to be attaching directly to the stainless steel at levels of 7.0 $\log_{10}$ cells cm^{-2} (Bouman *et al.,* 1982). In pilot scale plant, biofilms of thermo-resistant streptococci reached a steady state after approximately 12 h at 42.5°C when the growth and detachment rates were equivalent (4.9 $\log_{10}$ cells cm^{-2} s^{-1}) (Lee *et al.,* 1997). Rademacher *et al.* (1995) reported that the adhesion and growth of thermophilic bacteria on the plate surfaces of a pasteuriser depended on the number of bacteria in the milk before pasteurisation and colonisation of a pasteuriser was often associated with thermal treatment (thermisation) of the milk before pasteurisation.

The potential for non starter lactic acid bacteria (NSLAB) (*e.g. Lactobacillus* species) contaminants in cheese to originate from biofilms was demonstrated by monitoring biofilm development on stainless steel chips inserted into cheese vats (Somers *et al.,* 1994a). The biofilms that developed were resistant to the routine cleaning regimes.

Table 2.1

Microorganisms involved in biofilm formation in different processes (adapted from Mucchetti, 1995)

Microorganisms	**Dairy process surface**
Acinetobacter	Milk transfer lines
Bacillus species	Ultrafiltration and reverse osmosis membranes, evaporators
Escherichia coli	Ultrafiltration membranes
Pseudomonas aeruginosa	Ultrafiltration membranes
Thermophilic non-spore-forming bacteria	Milk or whey evaporators - pre-warming section
Streptococcus thermophilus	Milk pasteurisation and cheese manufacture

2.2.2.2 Ultrafiltration of milk and whey

Bacillus species will form biofilms in ultrafiltration plants used to process milk or whey. *Bacillus licheniformis* was identified as the predominant bacterium colonising ultrafiltration plant for the standardisation of milk used in the manufacture of cheese (Lehmann, 1995). The development of a biofilm consisting of *Bacillus subtilis* on the

ultrafiltration membranes and stainless steel pipework of a plant processing whey (S. H. Flint and N. J. Hartley, unpublished data) resulted in the manufacture of whey powders containing thermophilic bacteria at > 4.0 $\log_{10}$ colony forming units g^{-1}. Biofilm growth eventually blocked the pores in the ultrafiltration membranes preventing further manufacture.

2.2.2.3 Milk powder manufacture

The growth of *Bacillus stearothermophilus* on the surface of a milk powder manufacturing plant has been recorded (Stadhouders *et al.,* 1982) and it is likely that many of the problems with contamination of milk powders with thermophilic bacteria are due to the detachment of microorganisms from biofilms that have developed on stainless steel surfaces. Thermophilic non-sporing bacteria identified as *Thermus thermophilus* (Klijn *et al.*, 1992) have been isolated from milk processing equipment used in the evaporation process (Langeveld *et al.,* 1990, 1995).

2.2.2.4 General milk processing operations

The predominant microflora on the surface of lines handling raw milk are Gram-negative psychrotrophic bacteria although the predominant genera in raw milk from the cow are Gram-positive bacteria (Lewis and Gilmour, 1987). Gram-negative bacteria enter the processing lines through water contamination. These readily adhere to surfaces and provide a potential source of contamination of the final product. Gram-negative bacteria have been reported to adhere more readily than Gram-positive bacteria (Speers and Gilmour, 1985; Suárez *et al.,* 1992; Criado *et al.,* 1994). Gram-negative bacteria are a concern to the manufacturer of dairy products because their proliferation up to the point of pasteurisation is a potential source of microbiological enzymes (proteases, lipases, phospholipases) that can alter milk composition and may result in organoleptic defects (Koutzayiotis, 1992).

Bacillus cereus, an important spoilage bacterium in pasteurised milk, readily adheres

to and colonises stainless steel, therefore providing a source of contamination that is not fully removed by routine cleaning (Te Giffel *et al.*, 1997).

Several different species of bacteria, originating from dairy manufacturing plant have the potential to form biofilms during the heat treatment of milk; however, the temperature of the wall of the manufacturing plant is an important factor determining the dominance of particular species (Langeveld *et al.*, 1995).

Although the contamination of milk from biofilm development in milk pasteurisers is the main concern, reduced milk flow and heat transfer have also been attributed to biofilm development in dairy manufacturing plant (Criado *et al.*, 1994).

2.3 METHODS TO DETECT BIOFILMS IN DAIRY MANUFACTURING PLANTS

Within a dairy manufacturing plant, the initial indication of a biofilm problem is normally manifested through difficulties experienced in maintaining the quality of the manufactured products. In a manufacturing plant, two methods are generally used to detect biofilms: (1) recovery by swabbing sections of the surface of the plant and subsequent plating of recovered cells on agar plates; (2) culture tests on clean water flushed through the plant. Techniques used to confirm a biofilm problem are often inadequate in that these tests rely on the removal of bacteria from sites by swabbing and bacterial enumeration by traditional dilution and plating techniques. Difficulties occur as the cells may be difficult to remove and methods relying on the culture of bacteria will fail to detect viable but non-culturable forms which may later threaten product quality. There is a need for rapid detection methods to provide manufacturers with a quick assessment of the hygiene of their manufacturing plant (Wong and Cerf, 1995). Techniques that show promise for the detection of bacteria in biofilms involve the detection of bacterial ATP, protein or polysaccharide on surfaces or in water flushed through the lines. Such rapid techniques will assist in monitoring the effectiveness of cleaning procedures.

2.4 CONTROL OF DAIRY BIOFILMS

2.4.1 Cleaning

Cleaning procedures used in dairy manufacturing plants are limited mainly to the use of cheap chemicals (caustic, acid and chlorine) and most sanitising regimes have remained unchanged since the early 1900s. The most significant development in cleaning was the concept of clean-in-place (CIP) systems which originated in the 1950s when manufacturing plants were smaller, less complex processes and the specifications were less stringent than they are today (Romney, 1990). A typical dairy manufacturing plant cleaning programme consists of a fresh water rinse, followed by 1.5% sodium hydroxide at 70-80°C, another water rinse, 0.5% nitric acid at 60°C, a water rinse and finally 200 ppm sodium hypochlorite at 10-20°C. Any modifications are usually attempts to reduce the cost of cleaning or to prevent deterioration of sensitive manufacturing plant components (*e.g.* ultrafiltration membranes).

In any manufacturing plant, the preferred cleaning system is one that meets the product quality criteria at the least cost (Dunsmore, 1980). Within the manufacturing plant, the microbial performance of a cleaning system will depend on the amount of soil accumulation. Dunsmore (1980) identified three phases in soil accumulation, *viz.* the increasing effect phase where soil is insufficient to protect microorganisms, the diminishing effect phase where soil accumulation starts to take effect and the constant effect phase where the cleaning system has reached equilibrium with the soiling system and the reduction in microbial numbers is constant for a given cleaning system. In most food manufacturing plants, it appears to be unlikely that a film would reach the final stage. However, as processing run times increase, the greater is the difficulty in cleaning the surfaces of manufacturing plant.

The design and performance of systems for cleaning product-contact surfaces of milking machines have been reviewed (Dunsmore *et al.*, 1981). The standard knowledge relating to cleaning systems (*e.g.* the importance of cleaning and

sanitising as complementary procedures) was emphasised as was the fact that the type of soil and the accumulation of soil over repeat soiling-washing cycles influence the effectiveness of cleaning and in turn affect the type of cleaning system used. This accumulation of soil over repeated cycles can be reduced by periodic alteration to the cleaning cycle, *i.e.* changing the type of detergent used. It is of interest that in dairy manufacturing plants the changing over of detergents frequently results in a short term increase in the numbers of bacteria detected in the product due to the removal of old soil and the exposure of bacteria that have been protected from cleaning agents. This problem may be reduced by increasing the frequency of the sanitiser change thereby preventing the accumulation of resistant cells. The intercycle period, *i.e.* the time between cleaning and the use of equipment, also impacts upon bacterial numbers, as considerable growth of bacteria can occur during the period of inactivity if moisture and some nutrients are available. Hence the favoured practice is to sanitise the equipment immediately before use or to hold sanitiser in the equipment during this intercycle period. The possibility of drying the equipment to prevent microbial growth during the intercycle period has not been reported. Other considerations in the contamination of equipment surfaces include the release of microorganisms from the surface during the passage of product or cleaning agents and the recontamination of downstream surfaces. However, this recontamination problem can be reduced by a reverse flow cleaning system. The number of bacteria attached to surfaces after soiling has been reported to be dependent on the number of adhesion sites available, the type of microorganism (Dunsmore *et al.*, 1981; Neu, 1992) and the amount of soiling (Dunsmore *et al.*, 1981).

A cold cleaning system for milking machines was proposed by Dunsmore *et al.* (1981). This relies on a soak stage during the intercycle period using an alkaline detergent sanitiser followed by a flush with a cold solution of acidic sanitiser prior to the start of milking. A hot (80°C) wash using an acid detergent followed by a caustic detergent is recommended periodically. However, this system is unlikely to be suitable for food processing plants where the intercycle period is short.

Detergents are not totally effective in eliminating bacteria. Acid products acting as a combined detergent and sanitiser are more effective in killing bacteria than are the alkaline sanitising products. Sanitisers also vary in their effectiveness in different situations. Chlorine sanitisers have been found to be more effective as pre-processing sanitisers whereas iodophor is more effective than chlorine if it is used post washing and is retained in the plant during the intercycle period.

Chlorine has the ability to remove, destabilise and disinfect a biofilm. However, rapid recovery of biofilms after chlorine treatment is often observed. This may be due to the rapid re-growth of surviving cells, the residual biofilm providing a conditioning layer for enhanced biofilm development, the removal of the polysaccharide component and exposure of cells to nutrients resulting in enhanced growth, or selection of resistant microorganisms (Characklis, 1990). Scanning electron microscopy studies have shown that sodium hypochlorite does not eliminate attached microorganisms and debris, with the adhesion fibrils clearly visible after treatment of the adhered cells with sodium hypochlorite (Schwach and Zottola, 1984; Stone and Zottola, 1985). Unfortunately no information on the pH used in these studies was given. Caustic chlorine washes are effective in biofilm removal but not very effective in killing either spores or vegetative cells. This is believed to be due to the caustic pH favouring hypochlorite ion-promoted detachment (Characklis, 1990). Acid conditions favour disinfection by hypochlorous acid. Alternating treatment between caustic (pH 8) and acid (pH 6.5) chlorine treatments may be an ideal combination for biofilm destruction and microbial inactivation respectively; however, this is not generally done in the dairy industry.

Wirtanen and Mattila-Sandholm (1992b) studied the effect of age on the resistance of biofilms to chlorine sanitiser using *Ps. fluorescens, L. monocytogenes* and *B. subtilis*. Two interesting results emerged: (1) a minimum of 48 h was necessary for the growth of a detectable biofilm, and (2) microorganisms were more resistant when the biofilm was produced in milk rather than meat media. In these trials, a clean surface was used at the start of the experiment. In a processing plant, it is

likely that the development of a biofilm will progress with each batch of product processed through the plant, particularly if the CIP system is deficient. A biofilm may therefore become detectable in a shorter time as the season progresses. The increased resistance of biofilm bacteria to sanitisers in the presence of milk was also reported by Somers *et al.* (1994b). In this study, biofilms of NSLAB, developed in the laboratory using skim milk, were shown to be more resistant to quaternary ammonium and chlorine based sanitisers than those developed in culture medium (Somers *et al.*, 1994b). This is obviously an important factor that should be investigated further in a dairy industry environment.

Both ozonation and chlorination were found to be effective in reducing populations of the milk spoilage species *Ps. fluorescens* and *Alcaligenes faecalis* on stainless steel surfaces by > 99% (Greene *et al.*, 1993). Ozone is believed to be a more powerful sanitiser than chlorine and is often used in the treatment of water supplies. It attacks the bacterial membrane at the glycoproteins, glycolipids or certain amino acids. It is effective against Gram-positive bacteria, including spore formers, as well as Gram-negative bacteria, viruses and amoebae.

Microbiologically produced products, such as the bacteriocin nisin, and biosurfactants have been reported to have activity against bacteria of concern to dairy manufacturers (Busscher *et al.*, 1994; Bower *et al.*, 1995; Daeschel, 1995), with biosurfactants suggested to have anti-adhesive properties (Pratt-Terpstra *et al.*, 1989; Busscher *et al.*, 1996).

2.4.2 Surfaces

Surface topography may be important in cleanability. The most commonly used finish in the dairy industry is unpolished 304 L stainless steel (2B) (Leclercq-Perlat and Lalande, 1994). This surface has been reported to be harder to clean than polished 2B samples (Masurovsky and Jordan, 1958; Leclercq-Perlat and Lalande, 1994). Although chemical polishing treatments produced a significant improvement in the

cleanability, repeated soiling and cleaning resulted in an increase in the number of bacteria retained on the surface (Masurovsky and Jordan, 1958; Leclercq-Perlat and Lalande, 1994).

The cleanability and surface characterisation of 304 L stainless steel were compared using RBS 35 ® surfactant as a reference to compare with the acidic (HNO_3) and caustic (NaOH) cleaning system commonly used in the dairy industry (Leclercq-Perlat and Lalande, 1994). Cleanability was assessed on the ability to remove *Bacillus stearothermophilus* spores which were counted in the rinsing and cleaning solutions and on the surface using an agar overlay technique. Differences in the chemical compositions of the surfaces were observed to affect the removal of *B. stearothermophilus* spores. Carbon, oxygen and iron concentrations were found to affect both soiling and the effectiveness of cleaning. These authors concluded that materials used in the manufacture of surfaces that resist chemical changes and detergents that do not change the composition of surfaces will be more hygienic than surfaces and detergents that interact producing chemical changes of the surface.

2.5 CONCLUSIONS

Clearly, any food industry conscious of costs would seek to use the most cost-effective cleaning and sanitising methods possible.

Increasingly stringent microbiological specifications for products, increases in manufacturing plant size and increases in lengths of production runs are all forcing food industries to re-assess critically their cleaning procedures. Improving our understanding of the nature and formation of biofilms is important in formulating methods for cost-effective plant cleaning and sanitising.

Five factors are involved in the development of a biofilm in a dairy manufacturing plant: (1) the type of microorganism, (2) the type of product being processed, (3) the operation conditions of the manufacturing plant (including length of operation and

temperature), (4) the type of surface and (5) the cleaning and sanitising regimes. The factors that are likely to be the easiest to control are the type of surface and cleaning regimes.

It may be possible to modify surfaces physically or chemically (*e.g.* electropolishing of stainless steel surfaces) to limit the adhesion of microorganisms. Strategies for preventing microbial adhesion to surfaces that have been successful in some fields include altering the surface chemistry (*e.g.* hydrophobicity or charge), treating with antimicrobial agents (*e.g.* antifoulant paints) or optimising equipment design (*e.g.* surface topography) (Bower *et al.*, 1996).

New techniques for bacterial control and biofilm removal that have recently been investigated focus mainly on detergents and sanitisers. These include trisodium phosphate (Somers *et al.*, 1994c), enzymes (Aldridge *et al.*, 1993), peracetic acid and hydrogen peroxide (Teichberg *et al.*, 1993), non-ionic and propoxylated surfactants (Humphries *et al.*, 1987), copper and chloride (Knox-Holmes, 1993), oxidising agents such as bromine, non oxidising biocides (*e.g.* glutaraldehyde) and chelating chemicals. Characklis (1990) has described mechanical methods of cleaning including the use of sponge (Taprogi) balls in pipelines. Continued research and development is likely in this area of detergent and sanitiser formulation, and in the design of cleaning strategies to remove biofilms and slow their development on food processing surfaces. This work is being spurred on by the commercial advantages to be gained by longer processing runs.

The product and the steps in its manufacture are important in determining the predominant bacterial types likely to be involved in biofilm formation on plant and equipment. The emphasis on control of biofilms in dairy systems has been on the *Pseudomonas* species and food borne pathogens. Other bacteria (*e.g.* *S. thermophilus*) have not been studied in such detail even though they form biofilms of concern to dairy manufacturing, resulting in the contamination of dairy products.

Although at least one study has considered the inactivation of spore formers (*e.g. B. subtilis*) in biofilms (Wirtanen and Mattila-Sandholm, 1992b) and one group has studied the adhesion mechanisms of bacterial spores (Husmark and Rönner, 1990, 1992; Rönner *et al.,* 1990). No detailed studies on the role of spores in the formation of biofilms have been reported and the effectiveness of current cleaning regimes on many of the species causing concern in food processing plants need to be assessed.

Further research is needed to design cleaning agents that penetrate and remove biofilms, and to formulate cleaning strategies that will slow the development of biofilms on food processing surfaces so that longer processing runs will be viable.

Chapter 3

MATERIALS AND METHODS

3.1 SOURCE OF ISOLATES

The cultures used in this study originated either from milk obtained during the manufacture of dairy products, stainless steel surfaces exposed to milk at 37°C for 12-18 h in the laboratory, biofilms present in dairy manufacturing plant or reference culture collections (Table 3.1). Isolates from industrial biofilms were obtained from samples taken from a modified Robbins device (MRD) (Section 3.3.1) installed in-line immediately downstream from the regeneration section of the pasteuriser.

3.2 CULTURE PREPARATION

For the routine preparation of cultures, all isolates were cultured in M17 broth and M17 agar (Difco, Fort Richard Laboratories, Auckland, New Zealand) (Terzaghi and Sandine, 1975). Composition: 5.0 g of Phytone peptone; 5.0 g of polypeptone; 3.5 g of yeast extract; 5.0 g of beef extract; 5.0 g of lactose; 0.5 g of ascorbic acid; 19 g of β-disodium glycerophosphate; 1 ml of 1.0 M $MgSO_4.7H_2O$; 1 l of deionised water. Cultures were grown aerobically at 37°C for 12-18 h.

Quantitative estimates of viable cells were prepared from the inoculation of M17 agar plates with 0.01 ml of serial 10-fold dilutions of cells prepared in sterile 0.75% NaCl (BDH Chemicals Ltd, Palmerston North, New Zealand). The plates were incubated at 37°C for 48 h and the number of colonies was counted.

Table 3.1

List of isolates of thermo-resistant streptococci and their origin

Strain number	Culture type	Source
21	*S. thermophilus*	Reference ATCC 19258
22	*S. thermophilus*	Reference NCTC 10353
39	*S. bovis*	Reference ATCC 33317
H	*S. thermophilus*	Pasteurised milk - cheese manufacture
L_3	*S. thermophilus*	Pasteurised milk - casein manufacture
L_4	*S. thermophilus*	Pasteurised milk - casein manufacture
BCD-7	*S. thermophilus*	Pasteurised milk - cheese manufacture
EF_2	*S. thermophilus*	Whey - cheese manufacture
36	*S. thermophilus*	Biofilm - milk pasteuriser, casein plant
37	*S. thermophilus*	Biofilm - milk pasteuriser, casein plant
38	*S. thermophilus*	Biofilm - milk pasteuriser, casein plant
41	*S. thermophilus*	Biofilm - milk pasteuriser, casein plant
42	*S. thermophilus*	Biofilm - milk pasteuriser, casein plant
46	*S. thermophilus*	Biofilm - milk pasteuriser, casein plant
47	*S. thermophilus*	Biofilm - milk pasteuriser, casein plant
48	*S. thermophilus*	Biofilm - milk pasteuriser, casein plant
3/1	*S. waiu* sp. nov.	Adhered cells - pasteurised milk
6/2	*S. waiu* sp. nov.	Adhered cells - pasteurised milk
7c	*S. waiu* sp. nov.	Adhered cells - pasteurised milk
T	*S. waiu* sp. nov.	Biofilm - milk pasteuriser, casein plant
43	*S. waiu* sp. nov.	Biofilm - milk pasteuriser, casein plant

3.3 BIOFILM DEVELOPMENT

3.3.1 Industry monitoring

In order to examine biofilm development and efficiency of cleaning procedures in a dairy manufacturing plant, a 304 stainless steel MRD with eight sampling ports was designed to be fitted into a 75 mm diameter stainless steel outlet pipe in an industrial plate heat exchanger pasteuriser (Figure 3.1). The design of the MRD was based on the original concept developed by Jim Robbins at the University of Calgary (McCoy *et al.*, 1981). Each of the sampling ports in the MRD was fitted with a stainless steel plug containing a removable 316 stainless steel coupon (10 mm diameter). The coupons were fitted flush with the internal surface of the pipe, and were used to analyse the development and removal of biofilms. The stainless steel coupons were cleaned by soaking and scrubbing in detergent (Cleanaid Powder (2 g l^{-1}); Intermed Scientific, Auckland, New Zealand) and sterilised by autoclaving (121°C for 15 min).

The MRD was inserted in line immediately following the regeneration section of a pasteuriser processing 25 000 l h^{-1} of skim milk. Temperatures in this region were recorded at 37-40°C. These are ideal temperatures for the growth of thermo-resistant streptococci. The pasteuriser processed the same amount of milk each week and was cleaned after each 6 h period of operation using a clean-in-place (CIP) system comprising a rinse with water, 0.8% NaOH, a rinse with water, 0.6% HNO_3 and a final rinse with water. The fluid at each step was above 70°C and each step was run for 45 min.

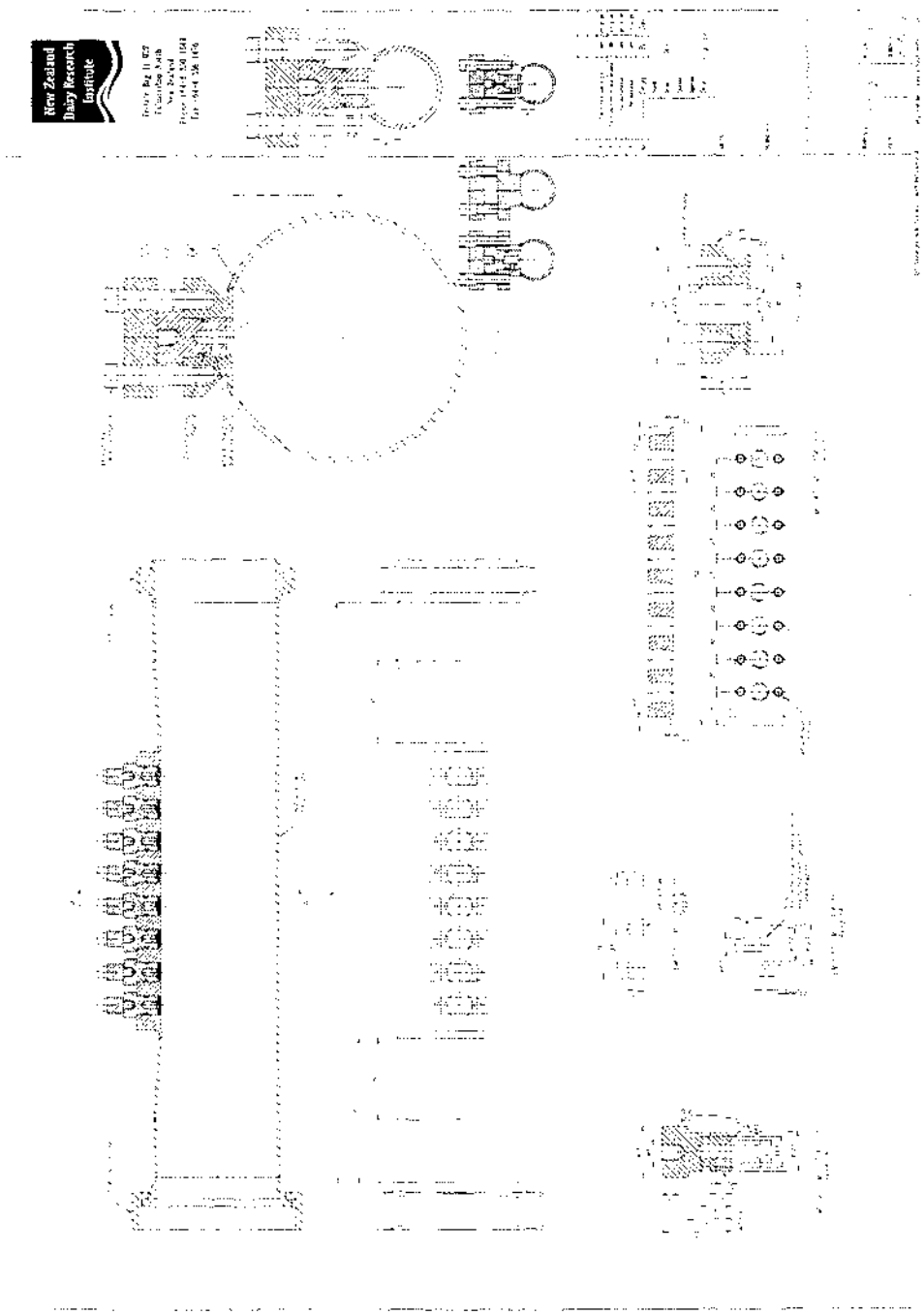


Figure 3.1 Design drawings for the MRD for sampling dairy manufacturing plant.

At weekly intervals, over 15 weeks, four coupons were removed from the MRD before and after cleaning and eight fresh coupons were aseptically installed. The total number of living and dead cells associated with the biofilm on the coupon surface was determined by a combination of epifluorescence microscopy (Section 3.4.1), fluorescent *in situ* hybridisation (Section 3.5.7) (two coupons examined before cleaning and two coupons examined after cleaning) and conductance detection (Section 3.4.2) (two coupons examined before cleaning and two coupons examined after cleaning).

3.3.2 Laboratory reactor

In order to produce biofilms on stainless steel surfaces in the laboratory for subsequent studies, a continuous flow reactor was designed, consisting of 9 mm internal diameter silicone tubing (Cole-Parmer, Niles, Illinois, USA) in which 1 cm^2 pieces of stainless steel were inserted to act as substrates for subsequent biofilm development (Figure 3.2). Coupons were equally spaced (maximum of five per 150 mm length of tube) along the length of the tube using sterile forceps and gentle manipulation down the tube using gentle pressure applied to the tubing with fingers and thumbs. The coupons were inoculated before insertion into the reactor tubing by exposing to 2 ml of washed cells (approximately 10^8 cells ml^{-1}) of thermo-resistant streptococci in 11 mm diameter test tubes for 30 min at ambient temperature (22°C), then washing with five changes of sterile deionised water. The tubing containing the inoculated coupons was connected to a recirculating system pumping pasteurised milk, at 140 ml min^{-1}, using a Masterflex peristaltic pump (Cole-Parmer, Niles, Illinois, USA), from a 250 ml flask in a water bath controlled at 37°C +/- 0.5°C. Fresh milk from a reservoir of fresh pasteurised milk kept in an adjacent room at 4°C +/- 0.5°C was added to the system using a multi-channel peristaltic pump (Gilson Minipulse 2, Villiers-le-bel, France). Milk was removed to waste at the same rate as

the addition of fresh milk using a separate line through the same pump. The rate of addition/removal of milk was adjusted to prevent a net increase in the number of bacteria due to growth in the milk. This required a rate that ensured that half the total volume in the system was replaced in the time taken for bacterial numbers to double. The doubling rate of the bacteria was estimated by taking hourly samples of bacteria in an M17 broth culture at 37°C and inoculating the surface of M17 agar plates with serial 10-fold dilutions of the samples in 0.1% peptone (Oxoid, Basingstoke, UK). Colonies appearing on the surface of the plates after 18 h incubation at 37°C were counted. The doubling time was calculated according to Pirt (1975) using the following formula:

$$td = \frac{Ln\ 2}{\mu}$$

td = doubling time

μ = specific growth rate

$$\mu = Ln\left(\frac{X}{X_0}\right) \cdot \frac{1}{t}$$

X = cell number at a point in late log phase of growth

X_0 = cell number at a point in early log phase of growth

t = total time between X and X_0

For the present work using *S. thermophilus* (H), the following two points were selected during logarithmic growth at 37°C to calculate the doubling time:

X = 1.7×10^6 colony forming units (cfu) ml^{-1}

X_0= 1.5×10^3 cfu ml^{-1}

Therefore μ = 1.75 and td = 0.396 h or 23.8 min.

The total volume in the reactor circuit was measured at 225 ml. Therefore 112.5 ml needed to be replaced within 23.8 min to ensure no increase in cells due to the growth of planktonic cells. Hence, the dilution rate required for the addition of fresh milk into the reactor was 4.7 ml min^{-1}.

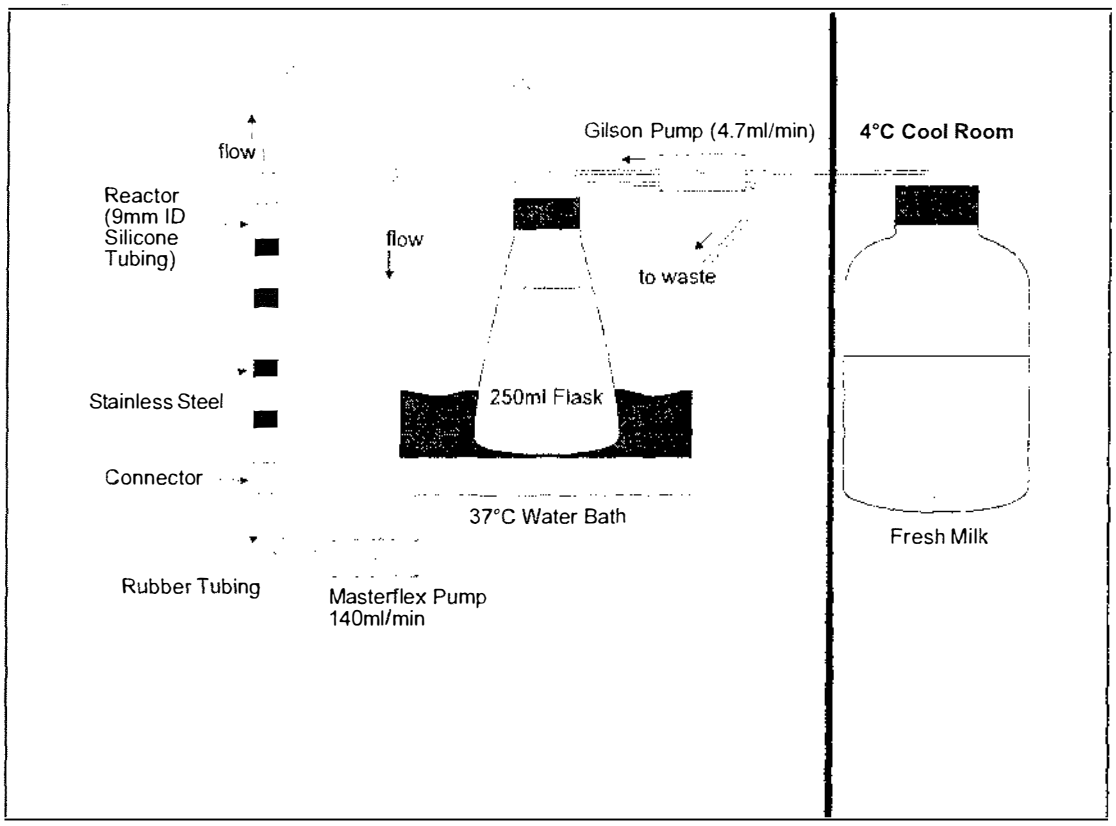


Figure 3.2 Schematic diagram of the laboratory biofilm reactor used to grow biofilms on 1 cm^2 coupons of stainless steel.

3.4 DETECTION METHODS

3.4.1 Epifluorescence microscopy

To determine the total number of cells colonising a surface, epifluorescence microscopy was used, based on the method of Ladd and Costerton (1990) with reference to a recent review by Kepner and Pratt (1994). The fluorochrome acridine orange (BDH) (10 mg) was dissolved in 100 ml of sterile phosphate buffer (0.1 M, pH 7.2) and filtered through a 0.2 μm Sartorius filter (Medic Corporation, Lower Hutt, New Zealand).

Samples were prepared for epifluorescence microscopy as follows. The specimens (samples supporting biofilms) were washed five times in sterile deionised water and then fixed with 1% formalin (BDH) at ambient temperature for at least 2 min. Samples were exposed to acridine orange for 2 min at ambient temperature, washed five times in sterile deionised water, air dried, mounted on glass microscope slides using epoxy resin (Araldite, Sellys, Auckland, New Zealand) and observed under ultraviolet (UV) light using a Leitz Ortholux II microscope with an H2 incident light excitation filter block (Ernst Leitz Wetzlar, GmbH, Wetzlar, Germany) and photographed using PJC 1600 film (Kodak, Rochester, New York, USA).

3.4.2 Conductance detection

To enable the rapid and sensitive detection of thermo-resistant streptococci as adhered or biofilm cells on the surface of 1 cm^2 samples of stainless steel, the Malthus microbiological growth analyser, AT model, (Malthus Instruments, Stoke-on-Trent, UK) was used. The Malthus enables the number of viable cells to be detected based on the time taken for a predetermined rate of change of conductance to occur

in the growth media. The detection time correlates well with the number of colony forming units (Brooks, 1986) and has been demonstrated to be a highly sensitive method to estimate the number of viable cells of thermo-resistant streptococci colonising stainless steel (Flint *et al.*, 1997a).

To prepare the Malthus apparatus for inoculation, the tubes and the electrodes were cleaned and sterilised by soaking in laboratory detergent (Cleanaid Powder, 2 g l^{-1}), gently brushing the electrodes, rinsing with five washes of deionised water and sterilising in deionised water by autoclaving (121°C for 15 min). The deionised water was discarded and the tubes were filled with sterile growth medium. This consisted of Colombia broth (BBL, Becton Dickinson and Co., Cockeysville, Maryland, USA), 1% skim milk (1.0 ml of a 10% solution of sterile skim milk added to 10 ml of Colombia broth) and 0.25% sucrose (0.1 ml of a filter-sterilised solution of sucrose (BDH) added to 10 ml of Colombia broth). The system was calibrated by inoculation of the tubes containing growth medium with 0.1 ml of serial 10-fold dilutions, prepared in 0.1% peptone, of *S. thermophilus* (H) from an 18 h culture. The same inocula were used to inoculate agar plates (M17) which were incubated at 37°C for 24 h. The number of colony forming units (cfu) was recorded together with the Malthus detection times required for a rate of change in conductivity of 10 μS h^{-1} during incubation at 37°C. A regression curve was prepared from these data using SigmaPlot (Jandel Corporation, San Rafael, California, USA) (Figure 3.3) and the following regression equation was used to estimate the number of viable bacteria:

$$Log_{10}\ cfu = -0.77 \times Detection\ Time\ (h) + 8.94$$

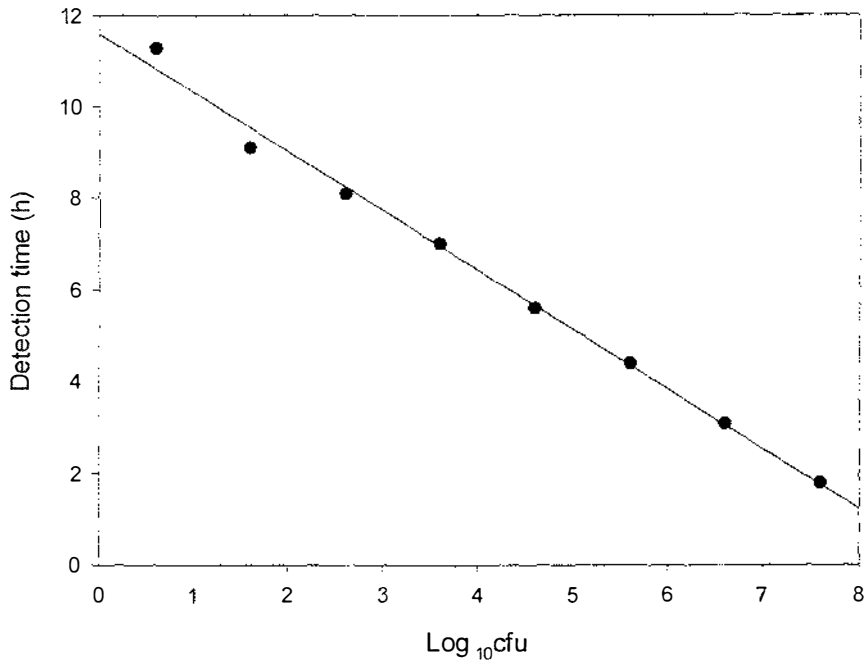


Figure 3.3 Regression calibration curve for the detection of *S. thermophilus* using the Malthus microbiological growth analyser (r^2 = 0.99).

3.4.3 Transmission electron microscopy

To visualise features of the cell surface, cells were examined by transmission electron microscopy (TEM). Sections of resin-embedded preparations were prepared from an 18 h culture of cells that had been centrifuged at 1000 *g* for 10 min and resuspended in deionised water. The cells were fixed at ambient temperature for 2-3 h in a primary fixative solution consisting of 3% (w/v) glutaraldehyde (BDH), 2% formaldehyde solution (BDH) in 0.1 M phosphate buffer (0.1 M KH_2PO_4 (BDH); 0.1 M Na_2HPO_4 (BDH)) at pH 7.2. The cells were washed with three changes of phosphate buffer, and exposed to a secondary fixative solution consisting of 1% osmium tetroxide (ProTech, Queensland, Australia) in 0.1 M phosphate buffer, pH 7.2 at ambient temperature for 30 min. After two washes in phosphate buffer, the cells were dehydrated using acetone (BDH) and embedded in Procure 812 epoxy resin (ProTech). Thin (90 nm) sections were cut using a microtome, stained with saturated uranyl acetate (BDH) in 50% ethanol followed by lead citrate (BDH) and examined under a Phillips 201C (Eindhoven, The Netherlands) transmission electron microscope.

To observe cell surface structures that might be responsible for adhesion, whole cells were treated with a negative stain. One drop of culture was placed on a 200 mesh Formvar-coated copper grid, followed by a drop of 1% phosphotungstic acid (Hopkins and Williams, London, UK), air dried and viewed in the Phillips 201 C transmission electron microscope.

To observe immunolabelled preparations (Section 3.6.12), samples were washed five times in sterile deionised water, and a drop of cell suspension was placed on a 200 mesh Formvar-coated copper grid and viewed under the electron microscope.

3.4.4 Direct counts of cell suspensions

To determine the total number of cells in suspension, serial 10-fold dilutions of cells were prepared in 0.1% peptone, and a drop of a dilution was placed in an improved Neubaur counting chamber (depth 0.1 mm, area of each counting square 0.0025 mm^2) (C. A. Hauser, Philadelphia, USA) and counted microscopically using an Olympus CHS microscope (Olympus, Tokyo, Japan) at 400 x magnification.

The following formula was used to determine the number of cells per millilitre of the sample:

$$\frac{X}{16} \; x \; 2.5 \; x \; 10^6$$

X = number of cells counted

16 = total number of counting chamber squares counted

2.5 x 10^6 = factor from the volume of each small square, multiplied by 100 to provide cells ml^{-1} .

3.5 BACTERIAL IDENTIFICATION AND CHARACTERISATION

To identify of the isolates of thermo-resistant streptococci from the dairy industry used in this work, a combination of morphological observations and biochemical and molecular assays was used. In addition, in an attempt to determine the potential of these isolates to form biofilms, they were characterised using a test for hydrophobicity.

3.5.1 Morphology and biochemical profile

Preliminary identification involved observing the colony morphology on M17 agar and BCP agar (Reddy *et al.*, 1969) (composition: 5.0 g of beef extract; 5.0 g of polypeptone; 5.0 g of phytone; 2.5 g of yeast extract; 0.5 g of ascorbic acid; 1.7 g of sodium acetate (anhydrous); 4.0 g of L-arginine hydrochloride; 2.0 g of lactose; 0.04 g of bromocresol purple dissolved in a small volume of 95% ethanol; 900 ml of deionised water; pH 6.8) and the Gram stain reaction of each isolate was observed. All isolates were also tested for the ability to grow in and clot reconstituted 10% skim milk (New Zealand Dairy Research Institute, Palmerston North, New Zealand) after incubation at 45°C for 12 h.

Typical thermo-resistant streptococci form pinpoint colonies in M17 agar, change the colour of BCP agar from blue to yellow due to the production of acid, appear as small (0.7 μm diameter) Gram-positive spherical cells in pairs and clot skim milk at 45°C.

The API 50 CH system (LaBalme Les Grottes, Montalieu, Vercieu, France) was used to provide information on the carbohydrate metabolism by following the manufacturer's instructions for inoculation, incubation and reading.

3.5.2 DNA extraction

To enable several experiments for the characterisation of thermo-resistant streptococci, genomic DNA was extracted using a procedure based on that of Marmur (1961). Cultures were prepared as described previously but incubated only until the mid-logarithmic phase of growth, previously determined by monitoring the optical density (OD) of cultures. Longer incubation times result in the degradation of DNA from thermo-resistant streptococci (Farrow *et al.*, 1984). Cells were harvested by

centrifugation at 9000 *g* for 10 min and resuspended in 30 ml of suspending buffer (50 mM Tris-hydrochloride (BDH) (pH 8.0), 1 mM EDTA and 0.35 M sucrose (BDH)) in a sterile glass-stoppered 250 ml bottle. Dry lysozyme (Sigma Chemical Co., St. Louis, Missouri, USA) (75 mg) was added to the suspension and incubated at 37°C for 30 min. 2-Mercaptoethanol (0.3 ml) (BDH), 0.7 ml of 0.5 M EDTA, 1.2 ml of 5 M NaCl and 100 µl of proteinase K (Boehringer Mannheim, New Zealand Ltd, Auckland, New Zealand) were added and swirled to mix. Sodium dodecyl sulphate (SDS) (BDH) (20% (w/v) in water) (1.5 ml) was added and the mixture was swirled to obtain uniform lysis of the cells. Lysis was indicated by an increase in the viscosity and a change in the suspension from turbid to opalescent. The lysate was incubated at 60°C for 60 min to allow the digestion of protein. Chloroform-isoamylalcohol (BDH) (24:1 v/v) (12 ml) was added and the mixture was shaken vigorously to obtain a homogeneous mixture. Shaking was continued on an orbital shaker for 20 min. The mixture was centrifuged at 17 000 *g* for 10 min and the upper aqueous layer was removed into a sterile glass beaker. This was overlaid with two volumes of cold (-20°C) 96% ethanol (BDH). The precipitated DNA at the ethanol-aqueous interface was collected with a sterile glass stirring rod (7 mm in diameter) by gently stirring the two phases while spinning the rod. The DNA adhered to the rod. Excess ethanol was removed by pressing the rod gently against the side of the beaker. The DNA on the rod was washed in 10 ml of cold (-20°C) 85% ethanol and the rod left inverted on a test tube rack to dry. The DNA was dissolved in 20 ml of 0.1 x standard saline citrate buffer (SSC) (0.015 M NaCl (BDH); 0.0015 M trisodium citrate (BDH); pH 7.0) by soaking in the buffer until the DNA loosened and could be slipped from the rod. After the DNA was completely dissolved, 1 ml of 20 x SSC (200 fold concentration of the 0.1 x SSC) was added to bring the concentration to approximately 1 x SSC. RNase A (1 mg ml^{-1}) (Sigma) (0.25 ml) and 500 units of T_1 RNase (Sigma) were added and the solution incubated at 37°C for 30 min. The DNA was placed in a ground-glass-stoppered 100 ml bottle and 5 ml of chloroform-isoamylalcohol added to the mixture and shaken at 37°C for 20 min. The mixture

was centrifuged at 17 000 *g* for 10 min and the aqueous layer was removed from the top. The DNA was precipitated as previously described and redissolved in 3 ml of 0.1 x SSC.

To determine the amount and purity of the extracted DNA, spectrophotometry and agarose gel electrophoresis were used. The amount of DNA was estimated by reading the OD at 260 nm using an Hitachi U 2000 spectrophotometer (Hitachi, Tokyo, Japan) calibrated with serial dilutions of calf thymus DNA (Sigma) (1.0 OD = 50 μg of DNA ml^{-1}). Purity was checked by inoculating 10 μl of sample with 1 μl of loading buffer (20% (v/v) Ficoll 400 (Sigma); 0.07% (w/v) bromophenol blue (BDH); 7.0% (w/v) SDS (BDH), on to a 2% agarose (Sigma) gel prepared in Tris/EDTA buffer (composition: 40 mM Tris (Sigma); 50 mM sodium acetate (BDH); 1 mM sodium ethylenediaminetetraacetate (EDTA) (BDH); pH 7.8) to which 20 μl of ethidium bromide solution (0.1 g of ethidium bromide (Gibco BRL, Auckland, New Zealand) in 10 ml of distilled water) was added. The gel was run at 80-100 V for 2-3 h until the tracking dye in the loading buffer reached the end of the gel. A 4 μl sample of a 1 kb DNA ladder (Gibco BRL) was included in one well. The DNA was observed under UV light and photographed using Polaroid 667 film (Polaroid, St Aubens, Hertfordshire, UK). Discrete bands indicated successful extraction of whole DNA. Any diffuse bands suggested DNA degradation and the extraction was repeated.

3.5.3 Restriction endonuclease analysis and ribotyping

To screen isolates for differences in their DNA composition, restriction endonuclease analysis (REA) and ribotyping (restriction patterns of DNA encoding rRNA) were used (Grimont and Grimont, 1991). Using REA, differences between isolates are determined visually by the pattern of fragments of DNA, following digestion with restriction enzymes, separated in an agarose gel. Ribotyping involves transferring

these fragments of DNA to a membrane and probing with labelled rRNA. This results in fewer bands being identified and allows easier interpretation of the results.

In this trial, DNA samples (2 μg) were added to microfuge tubes along with 2 μl of 10 x buffer (H) (Boehringer Mannheim), 2 μl of *ECO* RI (Boehringer Mannheim) and water to bring the final volume to 20 μl. The reaction mix was incubated at 37°C for 4 h and the total volume was separated in a 1% agarose gel as described previously (Section 3.5.2).

The separated DNA was transferred from the agarose gel to Hybond N+ membrane (Amersham International plc., Buckinghamshire, UK) by the method of Southern (1975). The gel was soaked in 0.25 M HCl for 30 min to fragment the DNA. The gel was rinsed with water to remove excess acid, the DNA was denatured by soaking the gel in 1.5 M NaCl and 0.5 M NaOH for 30 min. The gel was neutralised by soaking in precooled (0°C) 0.5 M Tris-HCL (pH 7.5). Two sheets of filter paper (Whatman, Kent, UK) 3 mm thick, saturated with blotting buffer (18 x SSC), were placed on a plane plastic block and the gel was placed on the filter paper. The Hybond membrane was cut to the size of the gel, floated on water, then soaked in blotting buffer before placing it on the top of the gel. Two sheets of filter paper saturated with blotting buffer were placed on top followed by 15 sheets of dry filter paper and a 2 cm stack of paper towels with a weight was placed on the top. Transfer was allowed to proceed overnight at 22°C. The membrane was then floated on 2 x SSC, then allowed to dry before baking in a vacuum oven at 80°C for 3 h.

For ribotyping, the DNA fragments transferred to the membrane were probed with an rRNA gene probe prepared by labelling a polymerase chain reaction (PCR) product amplified from the 16S rRNA gene (Section 3.5.6) using the enhanced chemiluminescent method (ECL, Amersham) with hybridisation and detection done according to the manufacturer's instructions, summarised below.

To prepare the probe, the DNA was labelled by diluting to 10 ng μl^{-1} in water and denaturing in boiling water for 5 min. An equal volume of labelling reagent (supplied with the kit) was added and the solution was mixed. The same volume of glutaraldehyde solution (supplied with the kit) was added and the mixture was mixed thoroughly and then incubated at 37°C for 10 min.

In preparation for hybridisation, 0.5 M NaCl was added to the ECL Gold hybridisation buffer at room temperature. The blocking agent (supplied with the kit) (5%) was added and the solution was mixed for 1 h and then preheated to 42°C for 1 h. The hybridisation buffer was added to the membrane containing the DNA fragments and allowed to prehybridise at 42°C for 15 min. The labelled probe was added and the solution was mixed gently and allowed to hybridise at 42°C overnight. The blots were washed twice in 0.5 x SSC, pH 7.0, with 0.4% SDS, at 55°C for 10 min. followed by two washings in 2 x SSC at 22°C for 5 min.

To detect the hybridisation reaction, equal volumes of the detection reagents 1 and 2 (supplied with the kit) were mixed. The wash solution was drained from the membranes and the detection solution was added to cover the membrane and left for 1 min. The solution was drained from the membrane and the membrane was then wrapped in plastic cling wrap and exposed to an X-ray film (Agfa, Belgium) at ambient temperature for 3 h. The image from the autoradiograph was used to interpret visually the results with a selection of bands from each isolate representing different sized fragments reacting with probe.

3.5.4 Random amplified polymorphic DNA (RAPD) analysis

To screen rapidly for the similarities between different isolates of thermo-resistant streptococci, RAPD analysis was used (Williams *et al.*, 1990). RAPD analysis uses the PCR to detect DNA polymorphisms by amplifying portions of genomic DNA

with single primers of arbitrary sequence which are short (9-10 bases) compared with traditional PCR primers (Williams *et al.,* 1990). This technique can be used to type any organism and has an advantage over many other molecular typing methods in that no DNA sequence information is needed. These arbitrary primers are used to exploit genetic variability by hybridising with compatible regions of genomic DNA and amplifying the regions where the primers are in the correct orientation. The amplification products can be visualised electrophoretically.

In this study, a random primer (RP (5'-CAGCACCCAC-3') (Dr. L. W. Ward, New Zealand Dairy Research Institute, Palmerston North, New Zealand) was used in a PCR to produce amplified gene products for comparison.

The PCR procedure used was based on that detailed in Taylor (1992). A reaction mix for 10 assays was prepared by combining the following: 460 µl of H_2O; 40 µl of 20 x buffer (TFL buffer, Epicentre Technologies, Madison, Wisconsin, USA); 80 µl of 25 mM $MgCl_2$; 160 µl of dNTP (Promega, Madison Wisconsin, USA); 1 µl of primer (Gibco BRL); 10 µl of TFL polymerase (Epicentre Technologies). The reaction mix was dispensed into reaction tubes (75 µl $tube^{-1}$) and template (culture) (5 µl) was added. DNA extraction is not required as sufficient cell lysis occurs in the reaction mix to allow the PCR reaction to preceed. The PCR reaction was allowed to take place under the following conditions: 1 cycle of denaturation at 94°C for 3 min; annealing at 45°C for 45 s; extension at 72°C for 1 min; followed by 30 cycles at 94°C for 45 s, 45°C for 45 s, and 72°C for 1 min, and one cycle at 94°C for 45 s, 45°C for 45 s and 72°C for 5 min.

Each completed PCR mixture (7.5 µl) was loaded on to a 2% agarose gel, separated and photographed as described previously (Section 3.5.2). The selections of bands representing different sized fragments of DNA from each isolate were compared.

3.5.5 Identification of *S. thermophilus, S. bovis* and *S. waiu* sp. nov. with PCR amplification using specific primers

To identify different isolates of thermo-resistant streptococci rapidly, PCR amplification using species-specific primers was used (Taylor, 1992). PCR is a technique for the *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. DNA polymerases carry out the synthesis of a complementary strand of DNA in the 5' to 3' direction using a single-stranded template, but starting from a double-stranded region. This is the primer extension reaction. The PCR uses the same principle but employs two primers, each complementary to opposite strands of the region of DNA, which have been denatured by heating. The primers are arranged so that each primer extension reaction directs the synthesis of DNA towards the other. Thus primer "A" directs the synthesis of a strand of DNA which can then be primed by primer "B" and *vice versa.* This results in the *de novo* synthesis of the region of DNA flanked by the two primers. The DNA in the region flanked by each primer will increase almost exponentially. The requirements for the reaction are simple: deoxynucleotides to provide the energy and nucleotides for the synthesis of DNA, DNA polymerase, primers, template (culture) and buffer containing magnesium. The deoxynucleotides and primers are present in excess so the synthesis can be repeated by heating the newly synthesised DNA to separate the strands and cooling to allow the primers to anneal to their complementary sequences.

To detect *S. thermophilus*, a specific primer for *S. thermophilus*, 23S therm (5' CATGCCTTCGCTTACGCT 3'), (Schleifer *et al.,* 1995) and a universal primer, 23 L (5' CGCAAACCGACAACAGGTAG 3'), were used in a PCR to determine if the isolates were *S. thermophilus* by the production of a PCR product from the gene encoding 23S rRNA (Schleifer *et al.,* 1995).

To detect *S. bovis,* the specific primer Bovis (5' CATCTAACATGTGTTAAATGC 3') (Dr. L. H. Ward, 1997, personal communication) was used in conjunction with the universal primer Y1 (5' TGGCTCAGAACGAACGCTGGCCCG 3') (Young *et al.,* 1991) to amplify a product from the 16S rRNA gene of *S. bovis.*

To detect *S. waiu,* a specific primer Waiu (5' GTCTCTAACATGTGTTAAACAC 3'), derived by comparing the partial sequence of 16S rRNA from this organism with other thermo-resistant streptococci, was used in conjunction with the universal primer Y1 to amplify a product from the 16S rRNA gene of *S. waiu* sp. nov.

The PCR conditions and preparation of the gel were as for Section 3.5.4 with the exception that the PCR was extended from 30 to 40 cycles. A positive result was indicated by the production of a band representing DNA amplified from the PCR reaction.

3.5.6 PCR amplification of DNA encoding 16S rRNA for partial sequence analysis

To provide further evidence for the classification of *S. waiu* as a new species, a partial sequence of DNA encoding 16S rRNA from this isolate was compared with the same region of the genome from other thermo-resistant streptococci. This technique has been used to help classify mesophilic lactic acid bacteria Klijn *et al.,*1991). The following pair of universal primers was used for the generation of oligonucleotides from DNA encoding the variable V2 region of 16S rRNA: Y1 and Y2 (5' CCCACTGCTGCCTCCCGTAGGAGT 3') (Young *et al.,* 1991). The same PCR conditions as previously were used. The DNA fragments produced were purified for sequence analysis using the Wizard Promega system (Promega Corporation, Madison, Wisconsin, USA). Sequence analysis was performed by Dr. Lawrence Ward at the New Zealand Dairy Research Institute. The sequence was compared with those

in the GenBank database.

3.5.7 Fluorescent *in situ* hybridisation

To enable *in situ* detection of *S. thermophilus* and *S. waiu* sp. nov., fluorescent probes were prepared. Fluorescent *in situ* hybridisation (FISH) using oligonucleotides conjugated to a fluorescent dye enables the direct identification of single cells by fluorescence microscopy (Amann *et al.*, 1990). The technique is useful for identifying bacteria in biofilms (Manz *et al.*, 1993). To prepare a *S. thermophilus* probe for FISH, a specific 23S rRNA sequence was selected from the published literature (Schleifer *et al.*, 1995) (5' CATGCCTTCGCTTACGCT 3'). For the *S. waiu* probe, a specific sequence encoding 16S rRNA was selected from a PCR amplified region by comparing the sequence with published sequences on a computer database (GenBank). The sequence selected for *S. waiu* was 22 base pairs, 12 of which were different from *S. thermophilus* (5' GTCTCTAACATGTGTTAAACAC 3'). The specificity of these sequences was confirmed by attempting to amplify (using PCR methods given above) the nucleic acid fragment containing this sequence from isolates of thermo-resistant streptococci. Probes were manufactured by Oligos Etc. (Wisconsonville, Oregon, USA) with a fluorescein label at the 5' end.

The protocol of Poulsen (1995) was used for FISH. Stainless steel samples with adhered cells of thermo-resistant streptococci were fixed in 4% formaldehyde (BDH) at 4°C overnight and dehydrated in an ethanol series (50, 80 and 96% ethanol for 3 min each). A hybridisation mix containing 40% formamide (BDH) and 2.5 ng of probe μl^{-1} was added to the surface of the sample and incubated at 37°C for 18 h in a humid chamber. The samples were washed three times with hybridisation solution without the probe, and then with a washing solution (1 M Tris pH 7.2 and 5 M NaCl (BDH)). Samples were dried, mounted on glass microscope slides as described earlier (Section 3.4.1) and observed under epifluorescence microscopy using a Leitz

Ortholux II with an I2 incident light filter producing an excitation wavelength of 450-490 nm. A positive result produced fluorescently labelled cells that were easily counted microscopically.

3.5.8 DNA/DNA hybridisation

To determine the relationship between the DNA from different isolates of thermo-resistant streptococci, DNA/DNA hybridisation was carried out using DNA from *S. thermophilus* (21), *S. bovis* (39), and *S. waiu* sp. nov. (3/1). DNA/DNA hybridisation is widely used to measure relationships within and between bacterial species (Stakebrandt and Goodfellow, 1991). In this study, the method used was based on that of Garvie (1976). The DNA to be tested (1 μg ml^{-1}, 100 μl) was heated to 100°C, chilled on ice and 1 volume of 20 x SSC was added. Serial two-fold dilutions were prepared in 10 x SSC to provide concentrations of 0.125-1 μg DNA in 200 μl. Nylon blotting membrane (Hybond N) was pre wetted with 10 x SSC for 5 min, then placed in a dot blot filtration manifold system (Gibco BRL) and 200 μl samples of each dilution were loaded into the wells of the blotting apparatus. The membrane was inoculated by pulling a vacuum to draw the samples through the membrane, removed from the blotting apparatus and placed in denaturing solution (1.0 M NaOH; 3.0 M NaCl), followed by neutralising solution (0.5 M Tris HCl, pH 7.5; 1.5 M NaCl) at ambient temperature for 5 min each before being placed between two sheets of blotting paper and air dried. The DNA was fixed by baking in a vacuum oven at 80°C for 2 h. The membranes were placed in glass hybridisation tubes with 10 ml of Rapid-hyb solution (Amersham), pre-warmed to 65°C and incubated at 65°C for 1 h.

To prepare the probe for hybridisation, DNA was diluted to 25 ng in 45 μl of sterile water in a microfuge tube and denatured by heating at 100°C for 5 min. The contents of the tube were centrifuged briefly (5 s in a microfuge) to bring the contents to the

bottom of the tube. The denatured DNA was then added to the labelling mix (Rediprime DNA labelling system (Amersham)) and mixed by gently flicking the tube until the blue colour was evenly distributed. The tube was centrifuged briefly and 5 μl of Redivue [^{32}P] dCTP (Amersham) was added and mixed by gently pipetting up and down 4-5 times. This mixture was centrifuged briefly and then incubated at 37°C for 10 min. The reaction was stopped and the probe was denatured by heating at 100°C for 5 min and then chilling on ice. The probe was added to the prehybridisation solution with the hybridisation membrane and incubated in a hybridisation oven at 65°C for 12 h. The hybridisation solution was removed and the membrane was washed twice with 2 x SSC at ambient temperature for 15 min and either once with 0.1 x SSC at ambient temperature for 15 min followed by 0.1 x SSC at 45°C for 15 min (high stringency) or twice with 0.5 x SSC at ambient temperature for 15 min (low stringency). The membrane was wrapped in plastic cling wrap and placed with an X-ray film (Agfa, Belgium) at ambient temperature for 3 h. The image from the autoradiograph was used to interpret visually the results (the size of the spots representing the degree of hybridisation) and to enable accurate cutting of the membrane for each hybridisation to be loaded separately into vials and the amount of ^{32}P associated with each hybridisation to be estimated using a scintillation counter (Packard Tri Carb 1900 TR Liquid Scintillation Counter, Illinois, USA).

3.5.9 DNA base composition - guanine plus cytosine (G + C) content

To compare the DNA base composition of *S. waiu* with that of other thermo-resistant streptococci, the thermal denaturation temperature of DNA from *S. waiu* (3/1) was determined (Marmur and Doty, 1962). The DNA base composition (G + C content) is an important criterion in the classification of microorganisms. This is generally determined by the thermal denaturation temperature of the DNA.

In this trial, DNA (20 μg ml^{-1}) was placed in a quartz cuvette with a 1 cm light path,

and overlaid with paraffin (BDH) to prevent evaporation. The sample was placed in a Gilford spectrophotometer model 260 (Gilford Instrument Laboratories, Oberlin, Ohio, USA) with a sample chamber containing two thermal spacers on either side, enabling the temperature to be raised by circulating hot water. The temperature in the cuvette was monitored with a portable thermocouple inserted into a cuvette containing SSC. The temperature was raised to approximately 5°C below the estimated melting temperature (80°C), then slowly raised in 1°C increments and the OD was read at 260 nm. The OD at each temperature was divided by the value at 25°C and the ratio was plotted against temperature. The temperature corresponding to half the increase in the relative absorbance was recorded as the melting temperature (T_m). The G + C ratio was calculated from the formula:

$$G + C = \frac{T_m - 69.3}{0.41}$$

This equation was derived from the regression for G + C *vs* T_m in SSC (Marmur and Doty, 1962).

3.5.10 Hydrophobicity test

To characterise isolates, the relative cell surface hydrophobicity was determined using the microorganism adhesion to hydrocarbon (MATH) test (Rosenberg *et al.*, 1980) with modifications by Crow and Gopal (1995). Hydrophobicity is believed to play a major part in the adherence of many organisms (Denyer *et al.*, 1993). The results from this trial enabled studies of the effect of hydrophobicity on the development of biofilms of thermo-resistant streptococci.

In this trial, cultures were centrifuged at 3000 *g* for 10 min and the cells were resuspended in sterile deionised water to an OD_{600} of 1.2-1.6. Samples of each cell

suspension (3 ml) were added to 3 ml of xylene, mixed briefly on a vortex mixer, incubated at 30°C for 10 min and then mixed vigorously with a vortex mixer at ambient temperature for 2 min. The absorbance of the aqueous phase was measured at 600 nm after standing at ambient temperature for 20 min to allow phase separation. The percentage hydrophobicity was determined from the OD of the bacterial suspension (A_i) and the OD of the aqueous phase after mixing with xylene (A_f) using the formula:

$$\% \ h = \frac{A_i - A_f}{A_i} \times 100$$

3.6 ADHESION STUDIES

3.6.1 Adhesion method

To determine the ability of different isolates and differently treated samples of thermo-resistant streptococci to adhere to stainless steel surfaces and to prepare samples of stainless steel with recently adhered cells for experiments, stainless steel samples were exposed to suspensions of thermo-resistant streptococci. Preparation of the stainless steel coupons (1 cm^2) (316 grade) involved passivation (50% HNO_3, 70°C for 30 min followed by washing in deionised water) to ensure a clean surface, followed by sterilisation (121°C for 15 min), and placing in test tubes with washed cells of thermo-resistant streptococci (approximately 1 x 10^8 cells ml^{-1}) in deionised water at ambient temperature for 30 min. The coupons were rinsed five times in sterile deionised water and the number of cells adhering determined by epifluorescence microscopy (Section 3.4.1) or conductance detection (Section 3.4.2).

3.6.2 Inactivation of bacteria

To study the effect of cell viability on adhesion, cells of thermo-resistant streptococci were inactivated by three different methods. The cells were prepared from an 18 h culture of *S. thermophilus* (H) grown in M17 broth. The cells were harvested by centrifugation (10 000 *g* for 10 min) and resuspended in water. The cells were treated by one of the following methods.

(1) Heating to100°C for 10 min.
(2) Exposure to1% formalin (0.4% formaldehyde) at 22°C for 10 min.
(3) Exposure toUV light (254 nm) for 10 min.

The loss of viability was confirmed by spreading 0.1 ml of each on to the surface of M17 agar and the absence of colonies after incubating at 37°C for 48 h.

3.6.3 Cell surface charge test

To enable studies on the effect of cell surface charge on adhesion, the relative surface charge of thermo-resistant streptococci was tested by separating cells through anionic (Dowex AG 1 x 8 100-200 mesh) or cationic (Dowex AG 50W x 8 100-200 mesh) exchange resins (Bio-Rad Laboratories Ltd, Glenfield, Auckland, New Zealand) (Pedersen, 1980). Pasteur pipettes were plugged with glass wool and filled with 0.5 g of resin suspended in phosphate buffered saline (PBS) (5.0 g of NaCl; 7.0 g of Na_2HPO_4; 3.0 g of KH_2PO_4 in 1000 ml of deionised water; pH 7.0). The columns were eluted with 3 ml of PBS. The bacteria were prepared by centrifuging 5 ml of fresh culture (3000 *g* for 10 min) and resuspending in PBS to OD_{540} = 1.0. Each column was loaded with 1 ml of this suspension and eluted with 3 ml of PBS. The absorbance was measured at 540 nm and the percentage of bacteria bound to the resin (A_f) was calculated from the absorbance of a 1/4 dilution of the bacterial

suspension (A_0) and the absorbance of the sample from the column (A_1) using the following formula:

$$A_f = \frac{A_0 - A_1}{A_0} \times 100$$

3.6.4 Bacterial polysaccharide assay

To enable studies on the effect of cell surface polysaccharides on adhesion, the amount of extracellular polysaccharide (EPS) produced by each strain was determined. The acid hydrolysis method of Dall and Herndon (1989) described by Evans *et al.* (1994) was used. Cultures of thermo-resistant streptococci were centrifuged (3000 *g* for 10 min), resuspended in sterile deionised water to an OD_{600}= 1.2 (approximately equal to 8.0 $\log_{10}$ cells ml^{-1}) and sonicated (60 W for 2 min) in a sonicator water bath (Ultrasonics Pty, New South Wales, Australia). The samples were further centrifuged (1000 *g* for 10 min) to remove cells and the supernatant liquid was added dropwise to absolute ethanol (10 ml at 4°C). After standing at 4°C for 24 h, the precipitated EPS was collected by centrifugation (10 000 *g* for 20 min), resuspended in 1 ml of sterile water mixed with 7 ml of 77% H_2SO_4 (BDH) and transferred to a boiling tube in an ice bath for 10 min. Cold tryptophan (BDH) (1% w/v, 1 ml) was added and the tubes were heated in a boiling bath for 20 min to effect hydrolysis. Acid hydrolysis of EPS produces a furan which condenses with the tryptophan to produce a coloured product. This was quantified after cooling by measuring the OD_{500}. Calibration curves were prepared against standard dextran solutions (Figure 3.4). The results were expressed as dextran equivalent units 10^{-8} cells.

3.6.5 Removal of cell surface polysaccharides

To further investigate the effect of cell surface polysaccharides on adhesion, three chemical treatments were used to remove cell surface polysaccharides (Gopal and Reilly, 1995). Cultures of thermo-resistant streptococci were centrifuged (1000 *g* for 10 min) and the cells were resuspended to the original volume and treated in one of the following solutions: 10% trichloroacetic acid (TCA) (BDH) (100°C for 15 min); 50 mM sodium metaperiodate (BDH) (22°C for 34 h); 1% lysozyme (37°C for 60 min). After treatment, the cells were centrifuged (1000 *g* for 10 min) and resuspended in sterile deionised water.

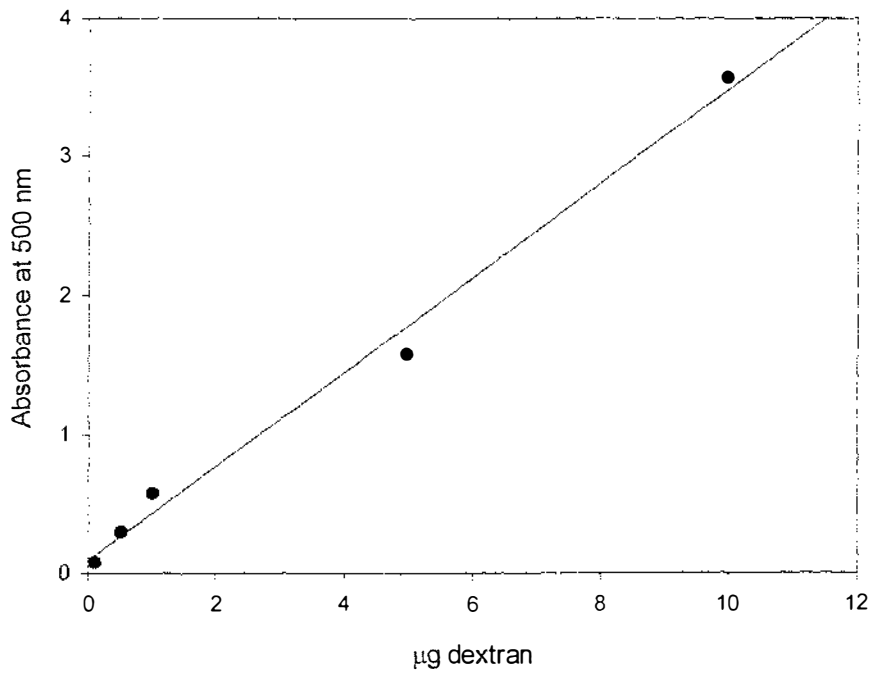


Figure 3.4 Calibration curve for the detection of EPS (r^2 = 0.99).

3.6.6 Removal of cell surface proteins

To enable studies on the effect of cell surface proteins on adhesion, two chemical treatments were used to remove cell surface proteins (Gopal and Reilly, 1995). Cultures of thermo-resistant streptococci were centrifuged (1000 *g* for 10 min) and the cells were resuspended to the original volume in the one of the following treatment solutions: either 1% trypsin (Sigma) (37°C for 24 h) or 2% SDS (100°C for 10 min). After treatment, the suspensions were centrifuged (1000 *g* for 10 min) and resuspended in sterile deionised water.

3.6.7 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS- PAGE) of cell proteins

To enable more detailed studies on the effect of treatments to remove cell surface proteins, samples of cells were tested using SDS-PAGE. The method used was based on Laemmli (1970) and detailed in the instructions provided with the Mighty Small II SE 250 electrophoresis system (Hoefer, San Francisco, California, USA). Stock solutions of the following were prepared.

(1) Monomer solution (58.4 g of acrylamide (Biorad, Hurcules, California, USA); 1.6 g of Bis (N, N'-methylene-bis-acrylamide) (Biorad); 200 ml of deionised water).

(2) Resolving gel buffer (36.3 g of Tris (Tris(hydroxymethyl)methylamine)); 200 ml of deionised water; pH 8.8)

(3) 10% SDS.

(4) Initiator (0.1 g of ammonium persulphate (Biorad); 1 ml of deionised water) - made up fresh, immediately before use.

(5) Treatment buffer (Tris (2.5 ml of a solution of 3.0 g of Tris; 50 ml of

deionised water; pH 6.8); SDS (4 ml of solution (3)); 2.0 ml of glycerol (BDH); 1 ml of 2-mercaptoethanol (BDH); 10 ml of deionised water).

(6) Tank buffer (12 g of Tris; 57.6 g of glycine (BDH); SDS (40 ml of solution (3)); 4 l of deionised water; pH 8.3).

(7) Stain (0.125 g of Coomassie Blue R 250 (BDH); 200 ml of methanol (BDH); 35 ml of acetic acid; deionised water to 500 ml).

(8) Destaining solution I (400 ml of methanol; 70 ml of acetic acid; deionised water to 1 l).

(9) Destaining solution II (70 ml of acetic acid; 50 ml of methanol; deionised water to 1 l).

Polyacrylamide gels (10% acrylamide) 1.5 mm thick were prepared by combining 3.33 ml of stock solution (1), 2.5 ml of solution (2), 0.4 ml of solution (3) and 16 ml of deionised water, in a conical side arm flask. This mixture was stirred with a magnetic stir bar on a magnetic stirring unit under a vacuum. After 3 min, the vacuum was disconnected and 50 µl of solution (4) and 5.0 µl of N,N,N'N'-tetramethylethylenediamine (TEMED) (Biorad) were added while mixing. The suspension was poured between glass plates separated with 1.5 mm spacers in the gel casting unit (Hoefer) and a 10 well comb was placed in the top of the gel. The gel was left overnight at ambient temperature to set. A stacking gel was not used as preliminary tests found this to be unnecessary to obtain clear separation of polypeptides.

Samples for SDS-PAGE separation were prepared from washed cell suspensions. Cultures (10 ml) were centrifuged and washed three times in sterile deionised water. The cells were suspended in 1 ml of deionised water in a conical test centrifuge tube (Falcon, Becton Dickenson, New Jersey, USA) following the final wash, lysed by adding approximately 2-3 g of glass beads (150-210 µm diameter) (Sigma) and mixing on a vortex mixer for 90 s. The lysis of the cells was confirmed

microscopically. The mixture was centrifuged, the supernatant was mixed with an equal volume of sample treatment buffer (solution (5)), boiled for 90 s and chilled on ice. The sample was either used immediately to load a well of the gel or frozen (-20°C) for future runs.

The 10-fold concentration of cells treated as given in this method normally produced samples containing 20-160 μg of protein in 0.02 ml. This was checked by testing samples for protein content using the method of Bradford (1976). The Bradford reagent was made by adding 100 mg of Coomassie Nile G (BDH) in 50 ml of 95% ethanol, adding 100 ml of 85% phosphoric acid (BDH) and diluting to 1000 ml with deionised water. Samples to be tested for protein content were prepared as above with the SDS treatment buffer replaced with 0.2 M NaOH. Note that SDS interferes with the Bradford assay and the treatment with NaOH simulates the treatment with SDS (Ionas, 1983). Each sample (0.1 ml) was mixed with 5 ml of the Bradford reagent and the absorbance at 595 nm was read after 2 min against a blank (0.1 ml of 0.2 M NaCl and 5 ml of reagent). The reading was compared with a standard curve prepared using serial dilutions of bovine serum albumin (BSA) (Sigma) (Figure 3.5).

The sample wells of the polyacrylamide gel were loaded with 0.02 ml of sample containing approximately 20-160 μg of protein. These samples were mixed with 0.001 ml of tracking dye (10% glycerol (BDH); 10% bromophenyl blue (BDH)) before addition to the wells. A 14 300-22 000 molecular weight protein standard (Rainbow Markers, Amersham) (2 μl) was included as a sample in each gel. Electrophoresis was carried out at 20 mA constant current for each gel. Once the tracking dye had reached the end of the gel, the gel was removed, placed in a square plastic petri dish (Biolab Scientific) and stained with Coomassie Blue R 250 with gentle shaking for 4 h. The gel was then destained with destaining solution I for 1 h followed by destaining solution II for 6 h. The gels were photographed using TMX

135 100ASA film (Kodak).

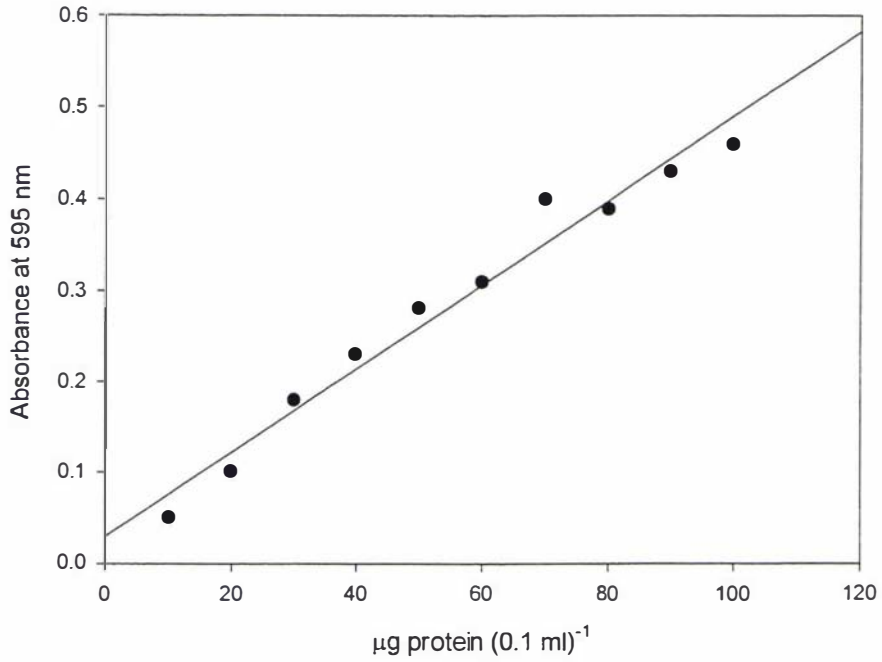


Figure 3.5 Calibration curve for protein estimation using the Bradford assay ($r^2 = 0.97$).

3.6.8 Western blot assays

To enable further analysis of proteins involved in adhesion, polypeptides from SDS-PAGE separation were transferred to a polyvinylidene difluoride (PVDF) membrane (Hoefer) using a method based on that of Towbin *et al.* (1979), Burnette (1981) and Tamura *et al.* (1985). To prepare the membrane for transfer, a sheet of PVDF membrane was cut to the size of the gel slab and marked in pencil with lanes for each sample. Each lane was numbered for future reference. The membrane was then soaked for 10 min in transfer buffer (0.13 g of potassium dihydrogen phosphate; 0.047 g of disodium hydrogen orthophosphate; made up to 1 l with 20% methanol; pH 6.5). Once SDS- PAGE separation was complete, one glass plate was removed and the gel was overlaid with a sheet of PVDF membrane. A clean glass rod was rolled over the membrane to remove any air trapped between the sheet and the gel. Filter paper, which had been moistened in transfer buffer, was laid on top of the membrane and a layer of synthetic sponge, which had been moistened in transfer buffer, was placed on top of this. The glass plate was removed from the other side of the gel and replaced with another sheet of moistened filter paper and synthetic sponge. This sandwich was placed between two porous plastic sheets from a transfer cell (Hoefer) and was inserted into the transfer cell with the membrane closest to the anode. The cell was filled with transfer buffer and the current was set at 300 mA. After 18 h, the membrane was removed, dried (37°C for 60 min) and cut with scissors along the lines separating the bands of electrophoresed protein. These strips were placed in square petri dishes for staining or probing. Additional strips were also stained as for the SDS-PAGE gels.

To probe membranes with specific antisera, the membranes were first washed in PBS containing 0.05% Tween 20 (BDH) (22°C for 5 min) followed by soaking in 5% BSA (22°C for 60 min) to block any sites on the membrane likely to cause non-specific binding of antisera or conjugates. The BSA was removed and the strips were

washed three times in PBS/Tween 20. Antisera diluted 10^{-1} in PBS/Tween 20 containing 1% BSA was added and allowed to react at 30°C on a rocker for 60 min. The membrane was washed five times in PBS/Tween 20, peroxidase anti-rabbit conjugate (Sigma) diluted $10^{-2.3}$ in PBS/Tween 20 was added and the membrane strips were incubated on the rocker (30°C for 60 min). The strips were washed three times in PBS/Tween 20. The substrate concentrate (2 ml) (30 mg of 4-chloro-1-naphthol in 10 ml of methanol) was added to 10 ml of substrate buffer (8 ml of 100 mM Tris HCl, pH 7.4; 8 ml of deionised water; 0.18 g of NaCl). This mixture was added to the membrane and allowed to react at 30°C in the dark for 15 min. The reaction was stopped by rinsing the membrane in tap water, and the strips were dried and photographed as for the SDS-PAGE gels.

3.6.9 Cell wall extracts

To enable studies of the cell wall components and their involvement in adhesion, loosely associated cell wall material was extracted by digesting cells with lysozyme and mutanolysin (Coolbear *et al.*, 1992; Crow *et al.*, 1993; Coolbear *et al.*, 1994). The cells from a 12 h culture of *S. thermophilus* (H) were harvested by centrifugation (10 000 *g* for 10 min), resuspended in sterile deionised water, centrifuged again and resuspended in the same volume of 24% sucrose in 50 mM Tris HCl, pH 7.0 with 10 mM $MgCl_2$. Lysozyme (Sigma) (1 mg ml^{-1}) and mutanolysin (90 units ml^{-1}) were added and the mixture was incubated at 37°C for 45 min. The treated cells were centrifuged (13 000 *g* for 10 min) at 20°C. The supernatant containing the cell wall fraction was concentrated 10-fold by ultrafiltration (20 000 molecular weight cut-off membrane) (Centrisart, Sartorius, Göttingen, Germany). The pellet containing the cytoplasmic fraction was resuspended to 10% of the original volume. Both fractions were analysed by SDS-PAGE.

To ensure that the cells had not ruptured during the treatment to remove the cell wall

proteins, the cell wall preparation was assayed to ensure the absence of the cytoplasmic enzyme β-galactosidase (Yu *et al.*, 1987). This consisted of mixing 0.9 ml of reaction buffer (0.1 M KH_2PO_4, pH 7.0; 0.002 M $MgCl_2$) with 50 µl of o-nitrophenyl β-D-galacto-pyranoside (ONPG) (Sigma) and a 50 µl sample of treated cells. The reaction was allowed to occur at room temperature with the presence of β-galactosidase indicated by a yellow colour change.

3.6.10 Antiserum preparation

To enable further studies on a specific protein of interest in adhesion, specific antiserum to this protein was prepared in rabbits. A 55 kDa polypeptide band associated with adhesion, and visualised by staining with Coomassie Blue, was cut from an SDS-PAGE gel using a scalpel, macerated in 1 ml of PBS by repeatedly forcing the gel fragment through a syringe fitted with an 18 G needle and mixed with an equal volume of Freunds' complete adjuvant (Difco), and an emulsion was prepared by repeated passage through the syringe. Once a complete emulsion had formed (determined by observing a discrete drop of emulsion that did not disperse on the surface of water), the entire volume was injected subcutaneously in 0.2 ml volumes at several sites into a New Zealand white rabbit. A second vaccine was prepared as described previously but replacing the Freunds' complete adjuvant with Freunds' incomplete adjuvant (Difco). This was injected 28 days following the first vaccination. A small (5 ml) sample of blood was taken from the marginal ear vein of the rabbit 14 days following the second vaccination. This was allowed to clot and the serum was separated by centrifugation. The serum was stored at -20°C until required for testing using the immunodiffusion assay and the bacterial agglutination test.

The immunodiffusion assay (Jurd, 1987) used 1% agar dissolved in deionised water and poured into a petri dish. Once this had set, wells, 5 mm in diameter, were cut

using a cork borer and a template consisting of a central well and six wells around the circumference with a distance of 2 mm between the outer wells and the central well. The agar was removed from the wells using a pasteur pipette attached to a vacuum pump. The central well was filled with a suspension of lysed cells of *S. thermophilus* and the outer wells were filled with serial 10-fold dilutions of antiserum from 10^{-1} to 10^{-6} and the plates were incubated (37°C for 48 h). The presence of antibodies was indicated by the development of a line of precipitation between the antibody and antigen wells.

The bacterial agglutination test was done in microtitre plates. Serial 10-fold dilutions of rabbit sera were prepared in 0.75% NaCl (BDH). Equal volumes of a cell suspension of *S. thermophilus* (H) in 0.75% NaCl were added to each dilution of sera. The microtitre plates were covered, incubated (37°C for 3 h) and examined microscopically for the agglutination of bacterial cells indicating the presence of agglutinating antiserum.

Following these initial tests, the same rabbits were inoculated intravenously with 1 ml of antigen prepared as described above, without the addition of adjuvant. A second blood sample was taken after 7 days and tested as described above. The vaccination and testing were repeated until no further increase in antibodies was detected by either method. A final 30 ml blood sample was taken from the marginal ear vein and the serum was extracted and stored as described previously.

3.6.11 Inhibition of adhesion using specific antibodies

To obtain further evidence for the involvement of a specific protein in adhesion, specific antibodies to the adhesion protein were used in an attempt to block adhesion. *S. thermophilus* (H) cells from an 18 h culture in M17 broth were harvested by centrifugation and resuspended in a 10^{-1} dilution of either specific antiserum to the

adhesion protein, or normal rabbit serum as a non-reactive control preparation. The cells were incubated with the antiserum (37°C for 1 h), centrifuged (10 000 *g* for 10 min) and resuspended in sterile deionised water. This centrifugation step was repeated three times to remove any unbound antiserum. Sterile coupons of stainless steel were exposed to these cells (22°C for 30 min), rinsed five times in sterile deionised water and stained with acridine orange (Section 3.4.1), and the number of cells adhering to the stainless steel were counted using epifluorescence microscopy and photographed (PJC 1600, Kodak).

3.6.12 Immunolabelling of cells using specific antibodies

To enable the specific proteins involved in adhesion to be observed on the cell surface, cells were immunolabelled with specific antibodies. Cells (10 ml) from an 18 h culture of *S. thermophilus* (H) were harvested by centrifugation (10 000 *g* for 10 min) and resuspended in a 10^{-1} dilution of antiserum in 1 ml of PBS with 0.5% BSA (PBS/BSA). After incubation (37°C for 1 h), the cells were washed three times in PBS/BSA and gold labelled anti-rabbit conjugate (Sigma) (10^{-1}) in PBS/BSA was added and incubated at 37°C for 1 h. The cells were washed three times with PBS/BSA with 5 min between washes and fixed in 0.5% glutaraldehyde (BDH) in preparation for examination by TEM (Section 3.4.3).

3.6.13 Preparation of a synthetic medium

To ensure an absence of milk proteins that may affect adhesion, a synthetic medium (Anderson and Elliker, 1953; Reiter and Oram, 1962) was prepared for the growth of thermo-resistant streptococci (Table 3.2). The complete medium (base, 990 ml; metal mixture, 10 ml) was autoclaved (121°C for 15 min).

Table 3.2

Composition of synthetic medium for the growth of thermo-resistant streptococci.

Component	Source	Quantity	Component	Source	Quantity
		$g\ l^{-1}$			$g\ l^{-1}$
Lactose	BDH	10	Ammonium citrate	BDH	0.6
K_2HPO_4	BDH	3	Sodium acetate	BDH	1
KH_2PO_4	BDH	3	Cysteine HCl	Sigma	0.5
D L-valine	Sigma	0.2	Ascorbic acid	BDH	0.5
L-histidine	Sigma	0.2	L-leucine	Signa	0.1
L-proline	Sigma	0.1	L-tryptophan	Sigma	0.2
L-aspartic acid	Sigma	0.5	L-glycine	Sigma	0.2
L-lysine HCl	Sigma	0.2	L-tyrosine	Sigma	0.1
L-serine	Sigma	0.2	L-cystine	Sigma	0.1
L-threonine	Sigma	0.2	DL ornithine	Sigma	0.6
Calcium pantothenate	Sigma	0.001	DL citruline	Sigma	2.6
L-glutamic acid	Sigma	0.5	L-alanine	Sigma	1.0
L-arginine HCl	Sigma	0.2	L-glutamine	Sigma	0.5
DL isoleucine	Sigma	0.2			$mg\ l^{-1}$
DL methionine	Sigma	0.2	Adenine	Sigma	5
DL phenylalanine	Sigma	0.2	Guanine	Sigma	5
		$\mu g\ l^{-1}$	Xanthine	Sigma	5
Nalidixic acid	Sigma	1	Uracil	Sigma	5
Para-aminobenzoic acid	Sigma	10	Pyridoxal hydrochloride	Sigma	2
Biotin	Sigma	10	Thiamine HCl	Sigma	1
Vitamin B_{12}	Sigma	1	Riboflavin	Sigma	1
Folinic acid	Sigma	1			
Folic acid	Sigma	1			
Metal mixture		$g\ l^{-1}$			$g\ l^{-1}$
$MgCl_2.6H_2O$	BDH	2	$ZnSO_4.7H_2O$	BDH	0.05
$CaCl_2$	BDH	0.5	$CoCl_2.6H_2O$	BDH	0.025
$FeCl_3.6H_2O$	BDH	0.05	$CuSO_4.5H_2O$	BDH	0.025

(Ref: Anderson and Elliker, 1953; Reiter and Oram, 1962)

3.6.14 Modification of M17 medium

In order to destroy or remove any proteins from M17 medium that may influence adhesion, the broth was subjected to either enzymatic digestion or ultrafiltration. M17 base medium was digested with 1% trypsin at 37°C for 48 h and then autoclaved (121°C for 15 min) to inactivate the enzyme and ensure sterility. A permeate of M17 medium was prepared by the ultrafiltration of M17 base medium through a 20 000 molecular weight cut-off membrane using the Centrisart ultrafilters (Sartorius).

3.6.15 The effect of β-lactoglobulin on adhesion

To determine the effect on adhesion of β-lactoglobulin (a milk protein very similar to the cell surface protein believed to be involved in adhesion of thermo-resistant streptococci) duplicate, clean, passivated, sterile, 316 grade stainless steel coupons (1 cm^2) were exposed to a sterile (membrane filtered) solution of 0.3% β-lactoglobulin at ambient temperature for 30 min. After rinsing five times in sterile deionised water, the coupons were exposed to a suspension of *S. thermophilus* (H) (1.0×10^8 cells ml^{-1}) at ambient temperature for 30 min, washed five times in sterile deionised water and the number of cells adhering was determined using the Malthus microbiological growh analyser. This was compared with the number of cells adhering to stainless steel coupons exposed only to the cell suspension. To ensure that cell viability was not affected by β-lactoglobulin, cells were exposed to 0.3% β-lactoglobulin for 30 min, and the cell numbers determined using the Malthus.

3.7 STUDIES ON THE REMOVAL AND INACTIVATION OF THERMO-RESISTANT STREPTOCOCCI

3.7.1 Heat treatment (Bremer and Osborne bag method)

To determine the effect of heat treatment on the viability of planktonic, adhered and biofilm cells of thermo-resistant streptococci, the following method, based on that of Bremer and Osborne (1997), was used. Samples for heat treatment (1 ml of cell suspension or 1 cm^2 stainless steel coupon in 1 ml of sterile deionised water) were placed into individual plastic bags (Whirl Pak, Biolab Scientific, Palmerston North, New Zealand). Air was removed from the bags by flattening the bags on a bench, ensuring no leakage, and the bags were sealed with the self sealing wire ties. String (1 m) was tied to each bag and the bags were fully immersed under a perforated stainless steel box in a heated water bath. The end of each string from each bag was placed outside the water bath to allow easy removal of the bag. The temperatures of the water bath and one extra bag, treated as a sample bag, were monitored with thermocouples attached to a chart recorder (Yokogawa, Tokyo, Japan). The treatment time was taken from the time taken for the sample bag containing the thermocouple to reach the temperature of the water bath. This lag time was approximately 30 s. The treatment time was monitored with a stopwatch. The bags were removed after the set exposure time and immersed in a beaker of cool (15°C) water for 60 s. Each bag was dried and swabbed with 95% ethanol. The bags were cut with scissors that had been sterilised in 95% ethanol and ignited to remove residual ethanol. Samples were aseptically removed using either a pipette (planktonic cells) or sterile forceps (stainless steel coupons) for analysis using conductance microbiology (Section 3.4.2). The data were analysed using regression analysis of cell numbers against the time of exposure to each temperature. The D-values (time required for a 10-fold reduction in the number of viable cells) were determined from the negative reciprocal of each

graph. The Z-values (temperature change (°C) required to produce a 10-fold change in the D-value) were determined from the negative reciprocal of the slope of a regression of $\log_{10}$D-value against temperature.

3.7.2 Heat treatment (tube method)

To compare the effect of different methods on the results for the heat treatment of cells adhered to surfaces, the following alternative test method was used. Samples to be treated (1.0 ml of cell suspension or a 1 cm^2 stainless steel coupon in 1 ml sterile deionised water) were placed into sterile test tubes (11 mm diameter) with caps. To prevent splashes avoiding heat treatment, care was taken during inoculation to dispense samples into the bottom of the tube.The tubes were immersed in a water bath and the temperatures monitored as in Section 3.7.1. The lag time was approximately 60 s. After the set exposure time, the tubes were removed and immersed in water (15°C) to cool before aseptically removing the samples for analysis using conductance microbiology (Section 3.4.2).

3.7.3 Sanitiser treatment

To determine the sensitivity of planktonic, adhered and biofilm cells to sodium hypochlorite (Clark Products, Napier, New Zealand) and cetyltrimethyl-ammoniumbromide (CTAB) (BDH), samples were exposed to concentrations of 0, 2, 10, 200, 400, 600, 800 and 1000 ppm of each sanitiser. These concentrations were chosen from preliminary tests and aimed to ensure that the concentrations of chlorine and quaternary ammonium sanitisers commonly used in the dairy industry (200 and 25 ppm respectively) were included in the trial.

The strength of sodium hypochlorite in the concentrated solution was determined by titration with sodium thiosulphate (Milk Industry Foundation, 1949). Potassium iodide

(10%) (20 ml) was dispensed into a 250 ml beaker and 5 ml of concentrated HCl was added slowly while stirring along with the sodium hypochlorite solution to be tested (1 ml) and 150 ml of deionised water. This solution was stirred continuously and sodium thiosulphate (0.1 M) was added from a burette until the brown colour of the solution changed to a pale yellow. Soluble starch solution (2 ml of a 2% solution) was added and the titration was continued until the solution became colourless. The concentration of free available chlorine (FAC) was determined by the following equation:

$$\%FAC = volume\ (ml)\ of\ 0.1\ M\ Na_2S_2O_3 \times 0.003546 \times 100$$

(1 ml of 0.1 M $Na_2S_2O_3$ = 0.003546 g FAC)

The pH of the sodium hypochlorite solution was adjusted before testing for FAC and use in the sensitivity trials to 6.8-7.0 by the addition of 1 M HCl. The sodium hypochlorite solution was diluted in sterile deionised water to provide the concentrations required for the experiment. Solutions were prepared fresh for each experiment. The CTAB concentrations were based on the weight of the product supplied and were diluted in sterile deionised water. All dilutions were prepared in 1 ml volumes in sterile test tubes.

Planktonic cells from 18 h cultures of *S. thermophilus* (H) or (48) were prepared for testing by centrifuging (1000 *g* for 10 min) the culture and resuspending in sterile deionised water. This was repeated three times to remove components of the growth medium. An aliquot of cell suspension (0.1 ml) was added to duplicate test tubes containing dilutions of either sanitiser as well as tubes containing sterile deionised water controls and mixed briefly on a vortex mixer. After exposure at 22°C for 30 min, the cells were washed three times by centrifugation (1000 *g* for 10 min), resuspended in sterile deionised water and sub-samples (0.1 ml) from each tube were

transferred to Malthus test tubes containing Col-M media. Inactivators were not used to stop the action of the sanitisers after the set exposure time as sodium thiosulphate, used to inactivate chlorine sanitisers, was found to be toxic to the cells and inactivators are not used in the sanitation of dairy manufacturing plant. The washing step was used to reduce the sanitiser concentration and the organic material in the Col-M growth medium was used to quench any residual sanitiser activity.

For tests on adhered (Section 3.6.1) or biofilm (Section 3.3.2) samples, duplicate stainless steel coupons containing the cells were prepared for testing by rinsing five times in sterile deionised water and adding to test tubes containing dilutions of sanitiser (as above). Coupons were removed after incubation at 22°C for 30 min and rinsed five times in sterile deionised water before adding to Malthus test tubes containing Col-M medium to detect viable cells.

3.7.4 Cleaning chemical laboratory trials

The effectiveness of different chemical treatments in removing thermo-resistant streptococci from the surface of stainless steel was determined. Duplicate coupons containing recently adhered cells (Section 3.6.1) or 4 or 18 h biofilms (Section 3.3.2) were placed in sterile test tubes and treated with 2 ml of the following solutions under the following conditions.

(1) Sterile deionised water- ambient temperature..
(2) 1% trypsin at 37°C for 24 h.
(3) 2% SDS at 100°C for 15 min.
(4) 10% TCA at 100°C for 15 min.
(5) 0.02% sodium metaperiodate at 22°C for 24 h.
(6) 1% lysozyme at 37°C for 1 h.
(7) 1 unit ml^{-1} mutanolysin for 37°C 1 h.

(8) 2% NaOH at 75°C for 30 min.

(9) 1.8% HNO_3 at 75°C for 30 min.

(10) Sterile deionised water at 100°C for 15 min.

Treated coupons were rinsed five times in sterile deionised water, stained with acridine orange (Section 3.4.1), and the cells were counted using epifluorescence microscopy.

3.7.5 Commercial enzyme-based cleaner ("Paradigm") laboratory trials

The effectiveness of a commercial enzyme-based cleaner in removing biofilms of thermo-resistant streptococci from stainless steel was determined in laboratory trials. Biofilms of *S. thermophilus* (H), grown on stainless steel coupons over 20 h in the laboratory reactor (Section 3.3.2), were cut from the reactor tubing using sterile scissors, aseptically added to test tubes and then washed five times with sterile deionised water. The coupons were treated with 2 ml of the following solutions under the following conditions.

(1) Sterile dionised water - ambient temperature.

(2) 1.6% NaOH at 75°C for 30 min.

(3) 0.08% "Paradigm 2010" (Ecolab Ltd, Hamilton, New Zealand) and 0.09% "Paradigm 2030" at 60°C for 30 min.

(4) Treatment (2) followed by treatment (3).

(5) 0.08% "Paradigm 2010" at 60°C for 30 min and then 0.09% "Paradigm 2030" at 60°C for 30 min.

(6) 0.08% "Paradigm 2010" and 0.09% "Paradigm 2030" at 60°C for 30 min and then 0.8% HNO_3 at 75°C for 30 min.

The cells remaining following treatment were examined by epifluorescence

microscopy (Section 3.4.1).

3.7.6 Commercial enzyme-based cleaner ("Paradigm") pilot plant trials

To confirm the effectiveness of the commercial enzyme-based cleaner in removing and inactivating biofilms of thermo-resistant streptococci from stainless steel surfaces, trials were carried out in a pilot plant (New Zealand Dairy Research Institute). The pilot plant pasteurisation equipment used for this trial consisted of three plate heat exchangers, one to preheat the milk to approximately 65°C, followed by a second pasteuriser heat exchanger in which the temperature was raised to 73°C and the milk was held for 15 s in holding tubes before regenerative cooling to 37°C. An MRD with eight sample ports, similar to that detailed in Figure 3.1 (but using a 25 mm diameter pipe), was located in-line following the pasteuriser. The milk was cooled for storage to < 7 °C in a third plate heat exchanger.

To inoculate the sample disks to be inserted into the MRD, the disks were first sterilised by autoclaving (121°C for 15 min) and placed in a sterile Teflon disk with eight depressions to hold all eight stainless steel disks. The Teflon disk, containing a magnetic stir bar to enable the unit to spin during the inoculation process to ensure even distribution of cells on the stainless steel sample disks, was placed in a sterile 150 ml beaker and placed on a magnetic stirring unit. The sample disks were exposed to a suspension of cells of *S. thermophilus* (H) containing approximately 8.0 $\log_{10}$ cells ml^{-1} in deionised water at 22°C for 30 min. The sample disks were then rinsed with sterile deionised water, removed from the Teflon disk and inserted, using sterile forceps, into the plugs of the MRD which in turn were aseptically inserted into the MRD.

The MRD was located immediately after the cooling section of the pasteuriser where the milk was at approximately 37°C. The pasteuriser was run for 8 h to allow

biofilms to develop on the inoculated surfaces of the MRD.

To determine the amount of biofilm cells on the sample disks, the pilot plant was rinsed with cool (15°C) water and four of the sample disks were removed from the MRD and replaced with sterile plugs. The total cell numbers were determined on two sample disks using epifluorescence microscopy (Section 3.4.1). Viable cell numbers were determined on the second pair of sample disks using conductance microbiology (Section 3.4.2). To avoid the possibility of contamination on the reverse side of the sample disks affecting the results, the reverse side of each sample disk was sanitised in 1% formalin followed by 95% ethanol and then rinsed in sterile deionised water before inoculating the Malthus tubes. Preliminary tests on inoculated disks established that this method of sanitising the reverse side of the disks did not inactivate cells on the test surface and did not result in any inhibition of growth in the Malthus tubes.

To determine the effect of different cleaning methods, the pilot plant was cleaned by four different procedures in separate experiments.

Run 1. 1.8% NaOH at 75°C for 30 min; water rinse; 1.0% HNO_3 at 75°C for 30 min; water rinse.

Run 2. 1.8% NaOH at 75°C for 30 min; water rinse.

Run 3. 0.08% Paradigm at 60°C for 30 min; water rinse; 1.8% NaOH at 75°C for 30 min.

Run 4. 0.08% Paradigm at 60°C for 30 min; water rinse; 1.0% HNO_3 at 75°C for 30 min.

The remaining four disks, which had not been removed before cleaning, were now removed from the MRD and examined as for the disks removed before cleaning.

3.8 ACCURACY AND REPRODUCIBILITY

Throughout this study, care was taken to ensure accurate and reproducible results. All tests were carried out in duplicate and, where appropriate, quantitative data were reported with standard deviations. Linear regressions were carried out using SigmaPlot (Jandel Corporation, San Rafael, California, USA). Equipment (*e.g.* the Malthus microbiological growth analyser) was calibrated on a regular basis.

Errors for incubation temperatures were +/- 0.5° C and for pH were +/- 0.1.

Chapter 4

ISOLATION, IDENTIFICATION AND HYDROPHOBICITY OF THERMO-RESISTANT STREPTOCOCCI FROM MILK AND DAIRY MANUFACTURING PLANT

4.1 INTRODUCTION

The genus *Streptococcus* consists of Gram-positive, spherical or ovoid cells that are typically arranged in chains or pairs. They are facultatively anaerobic, non-sporing, catalase-negative and homofermentative and have complex nutritional requirements (Hardie and Wiley, 1994). The aim of this study was to monitor the colonisation of stainless steel surfaces (biofilm development) with thermo-resistant streptococci in a dairy manufacturing plant and to identify and characterise these isolates and others from pasteurised milk and stainless steel surfaces exposed to pasteurised milk.

Thermo-resistant streptococci are characterised by their ability to tolerate pasteurisation (72°C for 15 s) and to grow at temperatures up to 52°C (Bridge and Smeath, 1983). The development of biofilms of thermo-resistant streptococci on the stainless steel surfaces of dairy manufacturing plant threatens the quality of dairy products (Flint *et al.*, 1997b). The detachment of bacteria comprising a biofilm and their release into the environment is part of the life cycle of any biofilm. Release of bacteria from biofilms in a milk pasteuriser has been recorded to result in levels of 10^6 cells ml^{-1} in the pasteurised milk (Driessen and Bouman, 1979). Thermo-resistant streptococci species isolated from dairy environments are often *S. thermophilus* or are referred to as "*S. thermophilus* like" organisms. The specific identification of thermo-resistant streptococci causing problems in the manufacture of dairy products would assist in identifying the source of the contamination and in developing methods to control contamination in dairy manufacture. As hydrophobicity of bacteria is associated with biofilm development (Oliveira, 1992), the hydrophobicity of these isolates may indicate whether biofilms are a likely origin.

The taxonomic status of *S. thermophilus* has varied over the years because of the close relationship between these streptococci and *S. salivarius*. *Streptococcus thermophilus* became *S. salivarius* subsp. *thermophilus* (Farrow and Collins, 1984)

until separate species status was reproposed by Schleifer *et al.* (1991) on the basis of genetic and phenetic criteria. Subsequently, 16S rRNA sequence data have demonstrated that the thermo-resistant streptococcus, *S. thermophilus,* is closely related to both *S. salivarius* and *S. vestibularis* (Wood and Holzapfel, 1995).

Streptococcus bovis is another thermo-resistant streptococcus and is associated with cattle and found in milk. Classification of this organism was hindered by the biochemical heterogeneity of the strains. Genetic studies confirmed that *S. bovis* was indeed made up of several distinct "species" (Farrow and Collins, 1984; Coykendell and Gustafson, 1985) although full descriptions of these have not been made. Of the three homology groups of *S. bovis* described in Wood and Holzapfel (1995), only one is currently represented by 16S rRNA sequence data in the GenBank database. In addition, information from DNA/DNA hybridisation has demonstrated a close relationship between *S. bovis* and *S. equinus* (Wood and Holzapfel, 1995).

The identity and hydrophobicity of thermo-resistant streptococci were determined for isolates from the surface of a dairy manufacturing plant exposed to pasteurised milk, or pasteurised milk from manufacturing plants producing products either contaminated with thermo-resistant streptococci or with no contamination problem. *Streptococcus waiu* sp. nov., isolated from the dairy industry, was described during this study.

4.2 PROCEDURES

Biofilms located in a dairy manufacturing plant after the pasteurser were monitored using a modified Robbins device (MRD) (Section 3.3.1), using epifluorescence microscopy (Section 3.4.1) or conductance microbiology using the Malthus microbiological growth analyser (Malthus) (Section 3.4.2) to detect the number of bacteria colonising the surfaces. The number of bacteria in the milk passing through the MRD was estimated using the aerobic plate count (APC) (Section 3.2) to test

milk samples taken at the start, middle and end of the manufacturing run. This monitoring of a manufacturing plant provided one source of isolates of thermo-resistant streptococci for identification and characterisation.

Other isolates of thermo-resistant streptococci were obtained from pasteurised milk from dairy manufacturing plants experiencing contamination with thermo-resistant streptococci, from stainless steel samples exposed (37°C for 12 h) to pasteurised milk from a manufacturing plant with no known problems with thermo-resistant streptococci and from reference cultures. A full list of the isolates used is given in Section 3.1.

Isolates were identified using standard microbiological techniques, biochemical profiles and molecular techniques (Section 3.5) and were tested to determine their cell surface hydrophobicity using the microorganism adhesion to hydrocarbon (MATH) test (Section 3.5.10).

4.3 RESULTS

4.3.1 Industry monitoring

There was little difference in the total number of cells associated with the surface of the stainless steel coupons taken from the MRD installed in a dairy manufacturing plant before and after routine cleaning (Figures 4.1, 4.2 and 4.3). In addition, the total number of cells detected on sterile sample surfaces installed in the MRD and left for one week in the manufacturing plant showed little variation over 15 weeks. Samples taken at week 7 were an exception, where cell numbers were almost two orders of magnitude higher, corresponding to processing a batch of poor quality milk (Figure 4.1).

The number of cells from the weekly samples taken from the surface of the sampling

unit before and after cleaning, and detected by the Malthus apparatus (Figures 4.2 and 4.3) was approximately 10-100-fold less than the total number of cells detected using epifluorescence microscopy (Figure 4.1) reflecting cell injury during the manufacturing and cleaning processes and/or some loss of viability during storage of the samples before testing.

The variations in the number of bacteria in the pasteurised milk detected by the APC (Figure 4.4) could not be explained by any corresponding variations in the number of bacteria detected in the biofilm samples. This may be explained by the fact that the APC detects all aerobic microflora, only some of which will be viable cells released from the biofilm.

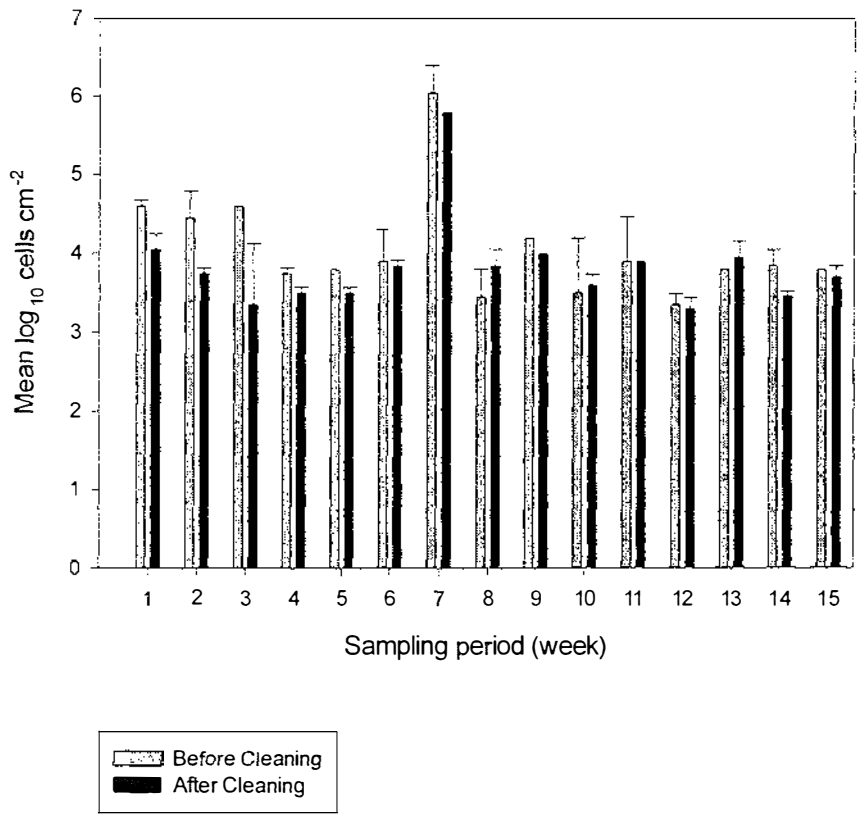


Figure 4.1 Total bacteria (mean and standard deviation from duplicate samples) determined by epifluorescence microscopy on test samples taken at weekly intervals from the MRD before and after cleaning.

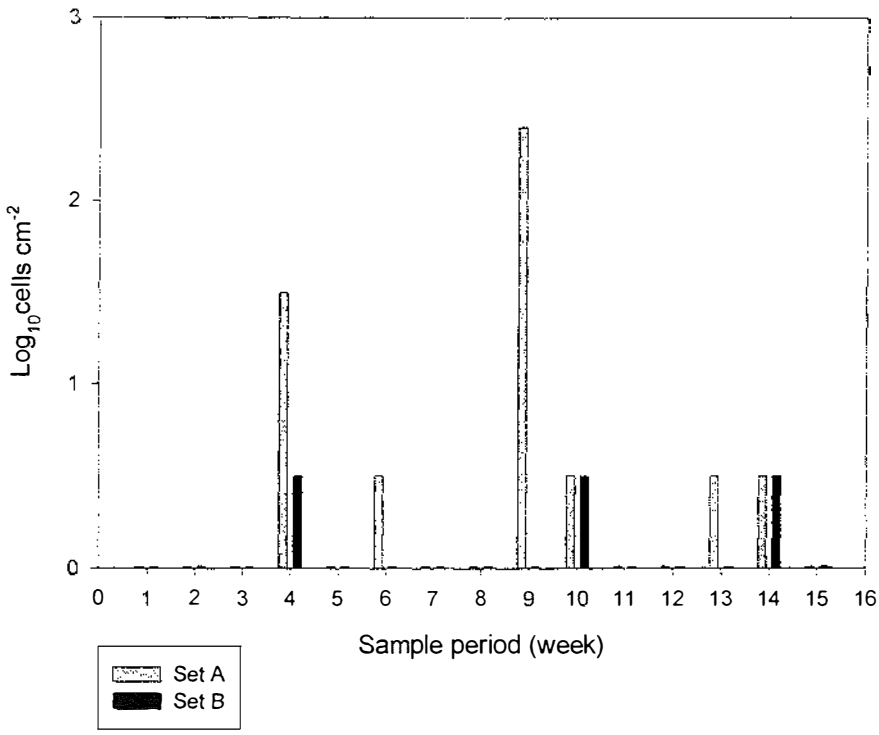


Figure 4.2 Viable cells detected in duplicate biofilm samples before cleaning using the Malthus. Where there are no data given for samples in any particular week, no viable cells were detected (with the Malthus, one cell on the stainless steel coupon can be detected). Sets A and B represent duplicate samples.

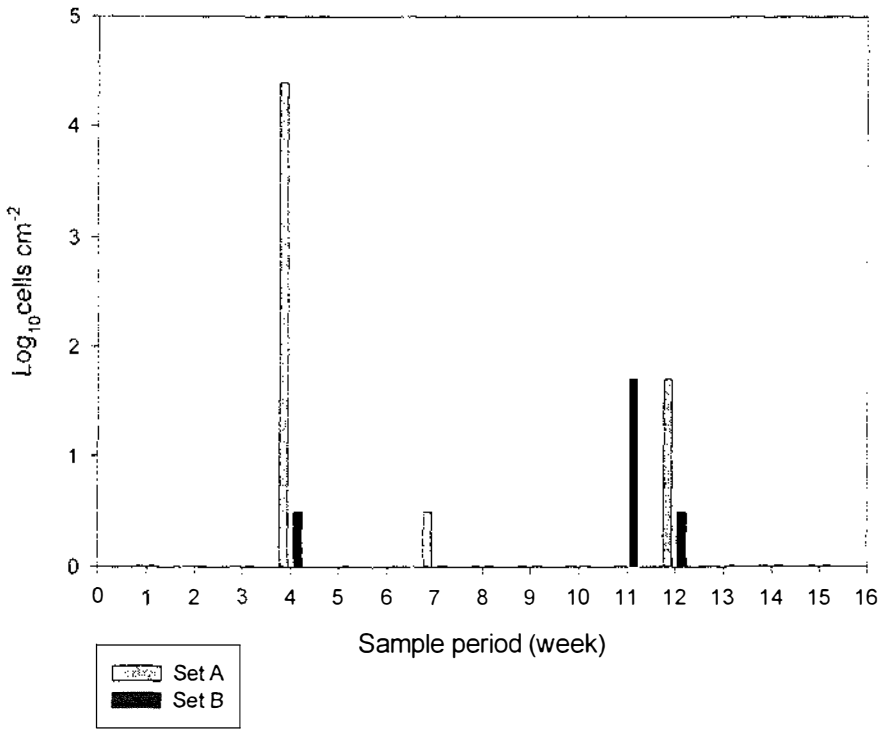


Figure 4.3 Viable cells detected in duplicate biofilm samples after cleaning using the Malthus. Where there are no data given for samples in any particular week, no viable cells were detected (with the Malthus, one cell on the stainless steel coupon can be detected). Sets A and B represent duplicate samples.

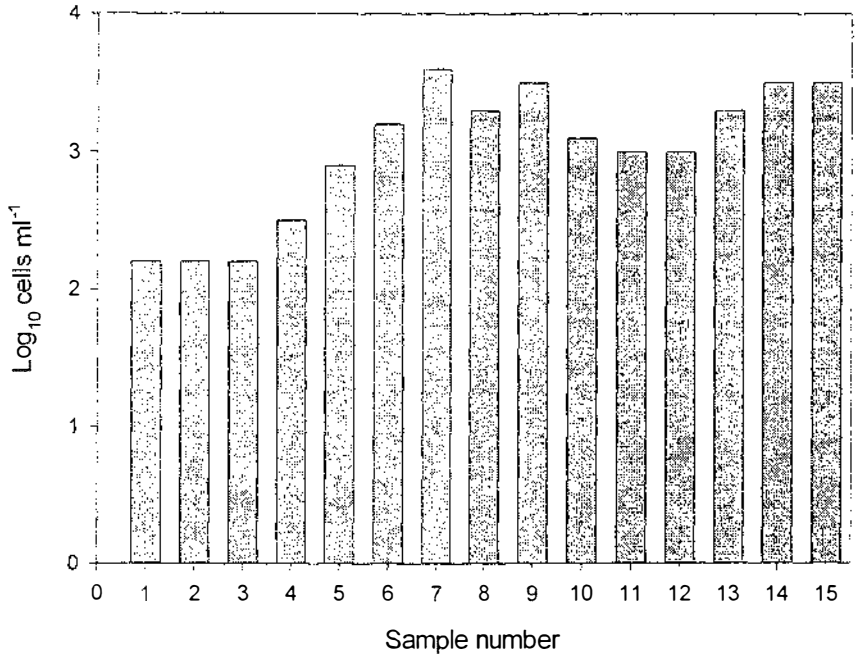


Figure 4.4 Total bacteria detected in the pasteurised milk using the APC during the 15 week sampling period.

4.3.2 Identification of isolates

4.3.2.1 Morphology and biochemical profiles

The isolates obtained from stainless steel surfaces in the dairy manufacturing plant were all typical of thermo-resistant streptococci. All the isolates produced pinpoint colonies on M17 agar, changed the colour of BCP agar from blue to yellow, indicating the production of acid from the fermentation of lactose, were small (0.7 μm diameter) Gram-positive spherical cells in pairs and clotted 10% reconstituted skim milk at 45°C. These features matched those of the reference strain of *S. thermophilus* (ATCC 19258). Isolates obtained from pasteurised milk from other manufacturing plants experiencing problems with contamination, isolates from pasteurised milk from manufacturing plants with no known problems and isolates from stainless steel surfaces exposed to pasteurised milk in the laboratory also shared these features.

The isolates were all tested using the API 50 CH test and five types were identified by the number of positive reactions (Table 4.1). These fell into two groups, one with a low number of positive reactions (4-7/49) (types I, II and III) represented by isolates from the surface of dairy manufacturing plant (36, 37, 38, 41, 42, 46, 47 and 48) and pasteurised milk from manufacturing plants reporting contamination problems (H, L_3, L_4, BCD-7 and EF_2). A second group consisting of isolates producing a higher number of positive reactions (12-13/49) (types IV and V) was represented by isolates from the surface of dairy manufacturing plant (43), pasteurised milk from a manufacturing plant reporting contamination problems (T) and from stainless steel samples exposed in the laboratory to pasteurised milk from a manufacturing plant with no known problems (3/1, 6/2 and 7c). The *S. thermophilus* reference strain (ATCC 19258) produced a positive reaction in 6/49 tests compared with 20/49 tests for the *S. bovis* reference strain (ATCC 33317). The isolates from the dairy industry

appeared to belong to either *S. thermophilus* represented by biochemical types I, II or III or another unknown species represented by biochemical types IV or V. None of the biochemical profiles of the isolates resembled the reference strain of *S. bovis*.

Table 4.1

Biochemical test profiles - thermo-resistant streptococci

Test	Reference culture or isolate type						
	thermophilus ref. ATCC 19258	*bovis* ref. ATCC 33317	Type I	Type II	Type III	Type IV	Type V
Glycerol	-	-	-	-	-	-	+
Erythritol	-	-	-	-	-	-	-
D-Arabinose	-	+	-	-	-	-	-
L-Arabinose	-	-	-	-	-	-	-
Ribose	-	-	-	-	-	-	-
D-Xylose	-	-	-	-	-	-	-
L-Xylose	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-
β-Methylxyloside	-	-	-	-	-	-	-
Galactose	-	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+
D-Fructose	+	+	-	+	+	+	+
D-Mannose	+	+	-	+	+	+	+
L-Sorbose	-	-	-	-	-	-	-
D-Rhamnose	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-
α-Methyl-D-mannoside	-	-	-	-	-	-	-

Note: Types refer to different biochemical profiles

Table 4.1 Continued

Test	*thermophilus*	*bovis*	Type I	Type II	Type III	Type IV	Type V
α-Methyl-D-glucoside	-	-	-	-	-	-	-
N-Acetyl glucosamine	+	+	-	-	-	+	+
Amygdaline	-	+	-	-	-	-	-
Arbutine	-	+	-	-	-	-	-
Esculin	-	+	-	-	-	-	-
Salacin	-	+	-	-	-	+	+
Cellobiose	-	+	-	-	-	+	+
Maltose	-	+	-	-	+	+	+
Lactose	+	+	+	+	+	+	+
Melibiose	-	+	-	-	-	+	+
Saccharose	+	+	+	+	+	+	+
Trehalose	-	-	-	-	-	-	-
Inulin	-	+	-	-	-	-	-
Melezitose	-	-	-	-	-	-	-
D-Raffinose	-	+	-	-	-	+	+
Amidon	-	+	-	-	-	-	-
Glycogene	-	+	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-
β-Gentibiose	-	+	-	-	-	-	-
D-Turanose	-	-	-	-	-	-	-
D-lyxose	-	-	-	-	-	-	-
D-Tagatose	-	-	-	-	-	-	-
D-Fructose	-	-	-	-	-	-	-
L-Fructose	-	-	-	-	-	-	-
D-Arabitol	-	-	-	-	-	-	-

Note: Types refer to different biochemical profiles

Table 4.1 Continued

Test	*thermophilus*	*bovis*	Type I	Type II	Type III	Type IV	Type V
L-Arabitol	-	-	-	-	-	-	-
Gluconate	-	-	-	-	-	-	-
2 Ceto-gluconate	-	-	-	-	-	-	-
5 Ceto-gluconate	-	-	-	-	-	-	-

Note: Types refer to different biochemical profiles

4.3.2.2 Restriction endonuclease analysis, ribotyping and random amplified polymorphic DNA analysis

To differentiate between different isolates of thermo-resistant streptococci, restriction endonuclease digestion with *Eco*R1, ribotyping and random amplified polymorphic DNA (RAPD) analysis distinguished three different profiles representing *S. thermophilus, S. bovis* and the unknown isolate subsequently named *Streptococcus waiu* sp. nov. (Figures 4.5 and 4.6A).

4.3.2.3 Identification of *S. thermolphilus, S. bovis* and *S. waiu* sp. nov. by PCR amplification using specific primers

To determine whether isolates were *S. thermophilus*, a DNA primer pair encoding 23S rRNA specific for *S. thermophilus,* was used in attempts to generate a polymerase chain reaction (PCR) product from each isolate. A PCR product (indicated by the presence of a band following gel electrophoresis) was successfully produced from isolates 36, 37, 38, 41, 42, 46, 47, 48, H, L_3, L_4, BCD-7 and EF_2 confirming that these isolates were *S. thermophilus.* No PCR product was produced from isolates 43, T, 7c, 3/1 and 6/2 indicating that these were not *S. thermophilus.*

To determine whether isolates were *S. bovis,* a DNA primer pair encoding 16S rRNA specific for *S. bovis* was used in attempts to generate a PCR product from each

isolate. No PCR product was produced from any of the 18 isolates listed above indicating that these were not *S. bovis.*

To determine whether isolates were the species subsquently named *S. waiu,* a DNA primer pair encoding 16S rRNA specific for *S. waiu,* derived from comparisons between partial sequences encoding 16S rRNA (Section 4.3.2.4), was used in attempts to generate a PCR product from each of the 18 isolates listed above. PCR products were produced from isolates 43, T, 7c, 3/1 and 6/2 indicating that these were *S. waiu.*

The results for isolates 3/1, 6/2, 7c, T, H and L3 with reference cultures *S. thermophilus* (ATCC 19258) and *S. bovis* (ATCC 33317) are given in Figure 4.6B, C and D.

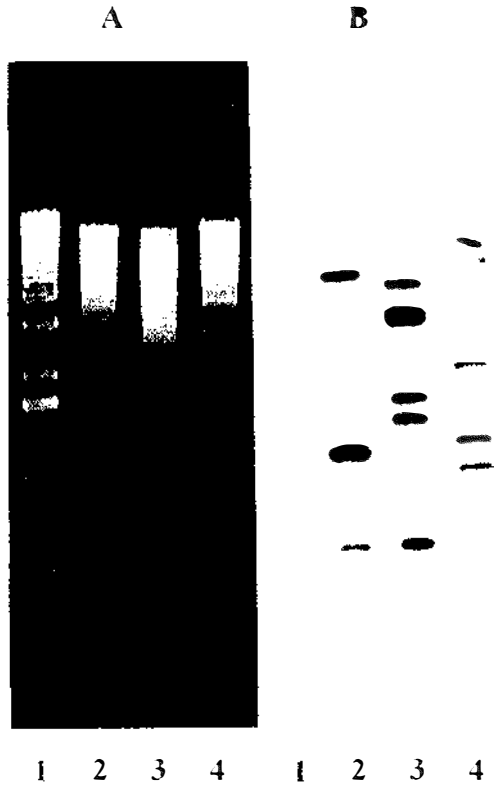


Figure 4.5 *Eco*R1 digestion of total DNA (A) and ribotype profiles (B). BRL 1 kb ladder (lane 1), *S. waiu* 3/1 (lane 2), *S. bovis* ATCC 33317 (lane 3), and *S. thermophilus* ATCC 19258 (lane 4).

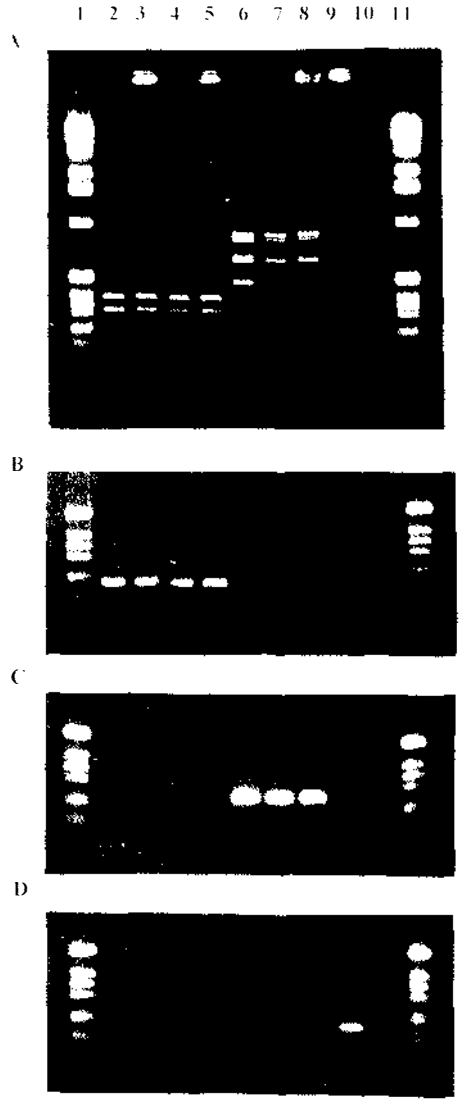


Figure 4.6 RAPD profiles (A), and PCR products obtained from amplification of total bacterial DNA with primer combinations specific for *S. waiu* (B), *S. thermophilus* (C) and *S. bovis* (D). Lanes 1 and 11, BRL 1 kb ladder; lane 2, *S. waiu* 3/1; lane 3, *S. waiu* 6/2; lane 4, *S. waiu* 7c; lane 5, *S. waiu* T; lane 6, *S. thermophilus* ATCC 19258; lane 7, *S. thermophilus* H; lane 8, *S. thermophilus* L3; lane 9, *S. bovis* ATCC 33317; lane 10, a negative control (all PCR reagents without any template (culture)).

4.3.2.4 PCR amplification of DNA encoding 16S rRNA for partial sequence analysis

To compare the unknown isolates of thermo-resistant streptococci with known species, partial sequences of a known variable region (V2) of the genome encoding 16S rRNA were compared. The universal primers Y1 and Y2 were used to produce a PCR product of 348 base pairs (bp) containing DNA from the variable region of the isolates that failed to produce a PCR product with the primer specific for *S. thermophilus*. The sequence of this product was aligned with the sequence of the same region of the 16S rRNA gene from four thermo-resistant streptococci (Figure 4.7). The percentage homology comparisons between these species (Table 4.2) demonstrated that the unknown isolates, named *Streptococcus waiu*, were most closely related to *S. equinus*. In the sequenced region, there were 12 bp different between *S. equinus* and *S. waiu*. This compares with two bp different between *S. bovis* and *S. equinus* and three bp different between *S. salivarius* and *S. thermophilus* in the same region. (There were 32 bp different between *S. waiu* and *S. thermophilus*.) The partial sequence of these unknown isolates, provisionally named *S. waiu,* has been lodged with GenBank under the accession number Bankit106530 U96621.

```
                1                                                      50
Thermophilus    TGGCTCAGGA  CGAACGGTGG  CGGCGTGCCT  AATACATGCA  AGTAGAACGC
Salivarius      TGGCTCAGGA  CGAACGCTGG  CGGCGTGCCT  AATACATGCA  AGTAGAACGC
Bovis           TGGCTCAGGA  CGAACGCTGG  CGGCGTGCCT  AATACATGCA  AGTAGAACGC
Equinus         ..........  ..........  ..........  ..........  ...
Waiu            ..GCTCAGAA  CGAACGCTGG  CGGCGTGCCT  AATACATGCA  AGTAGAACGC

                51                                                     100
Thermophilus    TGAAGAGAGG  AGCTTGCTCT  TCTTGGATGA  GTTGCGAACG  GGTGAGTAAC
Salivarius      TGAAGAGAGG  AGCTTGCTCT  TCTTGGATGA  GTTGCGAACG  GGTGAGTAAC
Bovis           TGAAGACTTT  AGCTTGCTAA  AGTTGGAAGA  GTTGCGAACG  GGTGAGTAAC
Equinus         ..........  ..........  ..........  ...GAACG  GGTGAGTNAC
Waiu            TGAAGACTTT  AGCTTGCTAG  AGTTGGAAGA  GTTGCGAACG  GGTGAGTAAC

                101                                                    150
Thermophilus    GCGTAGGTAA  CCTGCCTNGT  AGCGGGGGAT  AACTATTGGA  AACGATAGCT
Salivarius      GCGTAGGTAA  CCTGCCTTGT  AGCGGGGGAT  AACTATTGGA  AACGATAGCT
Bovis           GCGTAGGTAA  CCTGCCTACT  AGCGGGGGAT  AACTATTGGA  AACGATAGCT
Equinus         GCGTAGGTAA  CCTGCCTACT  AGCGGGGGAT  AACTNTTGGA  AACGATAGCT
Waiu            GCGTAGGTAA  CCTGCCTATT  AGTGGGGGAT  AACTATTGGA  AACGATAGCT

                151                                                    200
Thermophilus    AATACCGCAT  AACAATGGAT  GACACATGTC  ATTTATTTGA  AAGGGGCAAT
Salivarius      AATACCGCAT  AACAATGGAT  GACCCATGTC  ATTTATTTGA  AAGGGGCAAA
Bovis           AATACCGCAT  AACAGCATTT  AACACATGTT  AGATGCTTGA  AAGGAGCAAT
Equinus         NATACCGCAT  AACAGCATTT  AACACATGTT  AGATGCTTGA  AAGAAGCAAT
Waiu            AATACCGCAT  AATAGTGTTT  AACACATGTT  AGAGACTTAA  AAGATGCAAT
                            *******     **********  ******
                201                                                    250
Thermophilus    TGCTCCACTA  CAAGATGGAC  CTGCGTTGTA  TTAGCTAGTA  GGTGAGGTAA
Salivarius      TGCTCCACTA  CAAGATGGAC  CTGCGTTGTA  TTAGCTAGTA  GGTGAGGTAA
Bovis           TGCTTCACTA  GTAGATGGAC  CTGCGTTGTA  TTAGCTAGTT  GGTGAGGTAA
Equinus         TGCTTCACTA  GTAGATGGAC  CTNCGTNGTA  TTAGCTNGTT  GGTGAGGTAA
Waiu            TGCATCACTA  GTAGATGGAC  CTGCGTTGTA  TTAGCTAGTT  GGTGGGGTAA

                251                                                    300
Thermophilus    TGGCTTACCT  AGGCGACGAT  ACATAGCCGA  CCTGAGAGGG  TGATCGGCCA
Salivarius      CGGCTCACCT  AGGCGACGAT  ACATAGCCGA  CCTGAGAGGG  TGATCGGCCA
Bovis           CGGCTCACCA  AGGCGACGAT  ACATAGCCGA  CCTGAGAGGG  TGATCGGCCA
Equinus         AGGCTCACCA  AGGCGACGAT  ACATAGCCGA  CCTGAGAGGG  TNATCGGCCA
Waiu            CGGCCTACCA  AGGCGACGAT  ACATAGCCGA  CCTGAGAGGG  TGATCGGCCA

                301                                                    400
Thermophilus    CACTGGGACT  GAGACACGGC  CCAGACTCCT  ACGGGAGGCA  GCAGTAGGG
Salivarius      CACTGGGACT  GAGABABGGC  CCAGACTCCT  ACGGGAGGCA  GCAGTAGGG
Bovis           CACTGGGACT  CCAGACTCCT  CCAGACTCCT  ACGGGAGGCA  GCAGTAGGG
Equinus         CACTGGGACT  GAGACACGGC  CCNGACTCCT  ACGGGAGGCA  GCNGTAGGG
Waiu            CACTGGGACT  GAGACACGGC  CCAGACTCCT  ACGGGAGGCA  GCAGT...
```

Figure 4.7 Alignment of part of the 16S rRNA gene sequences from *S. thermophilus* (GenBank X68418), *S. salivarius* (GenBank M58839), *S. bovis* (GenBank X58317), *S. equinus* (GenBank X58318) and *S. waiu* sp. nov. The bases of *S. waiu* that are underlined differ from one or more of the other species.

* Denotes the 22 mer primer for *S. waiu.*

Table 4.2

Percentage homology for the variable region (348 bp) of the genes of thermo-resistant streptococci encoding 16S rRNA

	S. thermophilus	*S. salivarius*	*S. bovis*	*S. equinus*	*S. waiu*
S. thermophilus	100	99.2	91.7	91.6	89.1
S. salivarius		100	91.7	91.6	88.5
S. bovis			100	99.2	94.3
S. equinus				100	95.6

4.3.2.5 Fluorescent *in situ* hybridisation (FISH)

A fluorescein-labelled oligonucleotide probe specific for *S. waiu* was able to identify these biofilm isolates attached to stainless steel using FISH (Figure 4.8). This specific oligonucleotide did not react with *S. thermophilus* or *S. bovis* and therefore was able to differentiate *S. waiu* from other thermo-resistant streptococci associated with milk. A FISH probe made from DNA encoding 23S rRNA specific for *S. thermophilus* was able to differentiate between *S. thermophilus* and *S. bovis* or *S. waiu.*

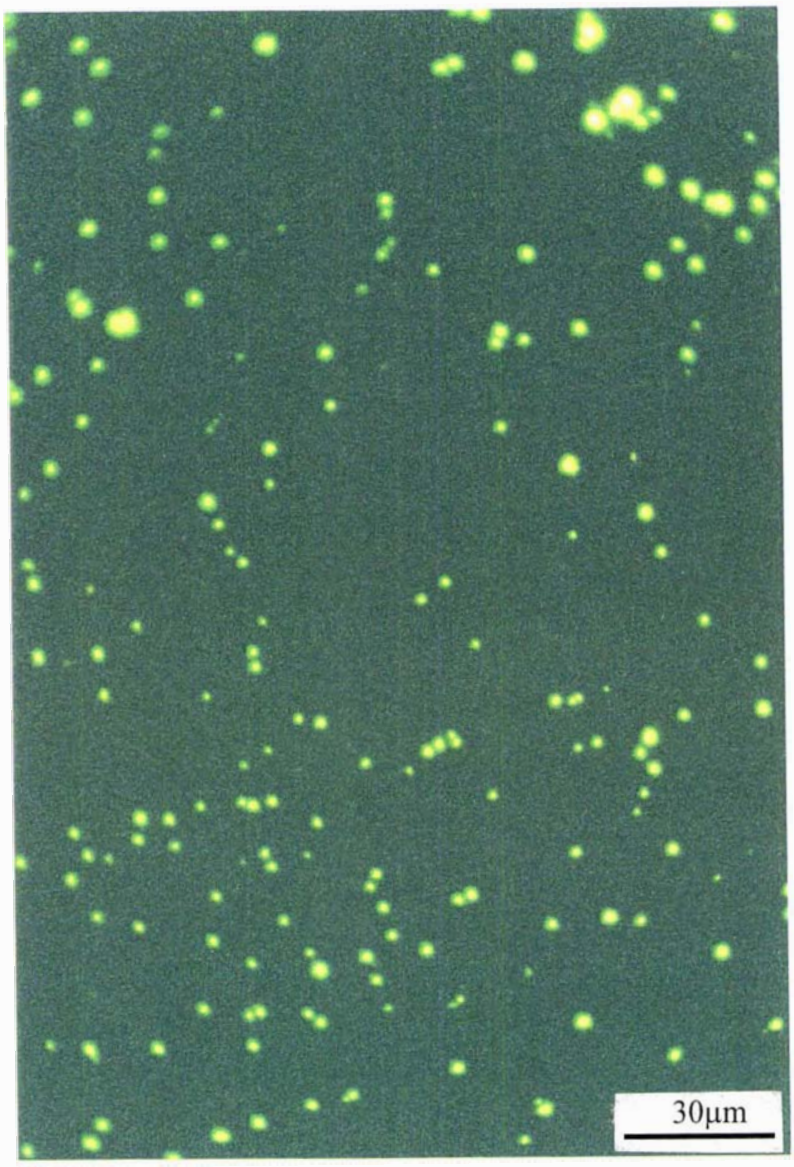


Figure 4.8 Fluorescent *in situ* hybridisation of *S. waiu* (3/1) using a 16S rRNA probe specific for *S. waiu*.

4.3.2.6 DNA composition

To confirm the identification of a new species and its relationship with other thermo-resistant streptococci, DNA/DNA hybridisation and the guanine + cytosine (G + C) content for three different isolates were examined. DNA/DNA hybridisation differentiated the three organisms and confirmed the results from the biochemical data, indicating a closer relationship between *S. waiu* and *S. bovis* than between *S. waiu* and *S. thermophilus* (Figure 4.9, Table 4.3). The G + C content for *S. waiu*, determined by the melting temperature (T_m), was 34.6%, adding further evidence to differentiate this species from *S. thermophilus* and *S. bovis* (Table 4.4).

Table 4.3

DNA/DNA hybridisation of *S. waiu* (3/1), *S. thermophilus* (ATCC 19258) and *S. bovis* (ATCC 33317) at high and low stringency

Probe	Homology (%)					
	High stringency			Low stringency		
	S. waiu	***S. therm.***	***S. bovis***	***S. waiu***	***S. therm.***	***S. bovis***
S. waiu	100	9.6	22.3	100	10.3	32.3
S. therm.	7.5	100	9.1	10.3	100	9.9
S. bovis	23.7	9.5	100	25.5	10.2	100

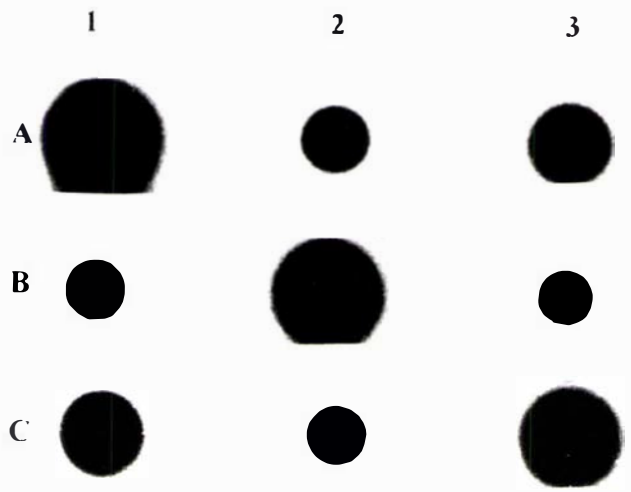


Figure 4.9 DNA/DNA hybridisation. Total DNA (0.125 µg) from *S. waiu* (3/1) (column 1), *S. thermophilus* (ATCC 19258) (column 2) and *S. bovis* (ATCC 33317) (column 3) was probed with ^{32}P-labelled total DNA from *S. waiu* (row A), *S. thermophilus* (row B) and *S. bovis* (row C). The dots represent the amount of radioactivity from the ^{32}P probes. The size of the dots indicate the amount of DNA probe binding to the DNA immobilised on the membrane.

Table 4.4

Summary of the G + C content determined from the melting temperature (T_m)

DNA source	T_m (°C) measured	T_m (°C) published	G + C (%) measured	G + C (%) published
Calf thymus	86.0	86.2	40.7	41.3
S. thermophilus	86.5	85.7	41.9	40.0
S. waiu	83.5	-	34.6	-

4.3.3 Cell surface hydrophobicity

The cell surface hydrophobicity of the thermo-resistant streptococci isolates was tested using the MATH test. All of the isolates originating from the biofilms or milk samples in dairy manufacturing plants experiencing problems with thermo-resistant streptococci were hydrophobic (Figures 4.10 and 4.11). Isolates from pasteurised skim milk from a manufacturing plant with no known problems and the reference cultures did not follow any trend in hydrophobicity (Figure 4.12).

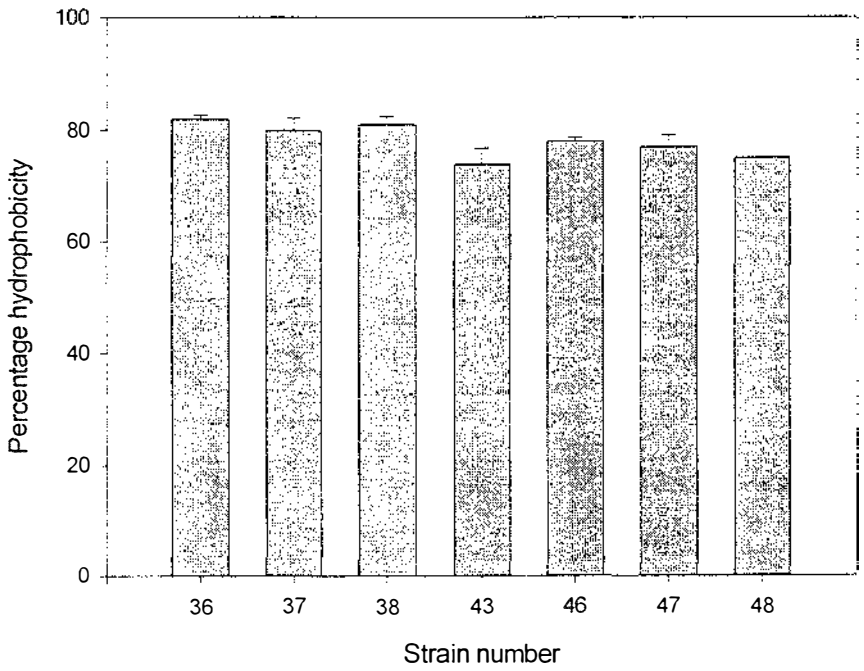


Figure 4.10 Hydrophobicity (mean and standard deviation from duplicate tests) of thermo-resistant streptococci isolated from biofilms in a dairy manufacturing plant.

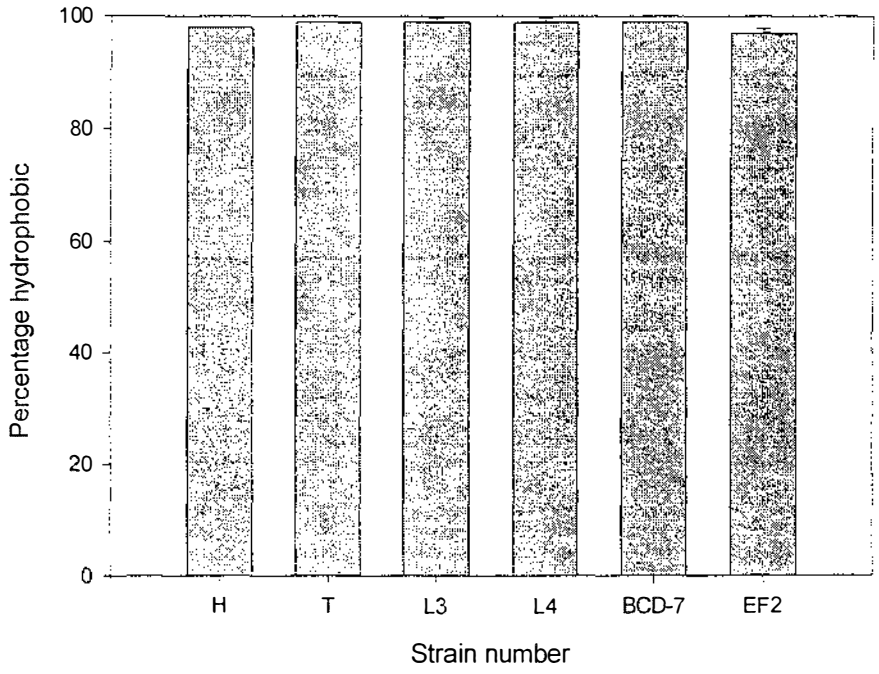


Figure 4.11 Hydrophobicity (mean and standard deviation from duplicate tests) of isolates of thermo-resistant streptococci from three different dairy manufacturing plants experiencing problems of contamination of products with thermo-resistant streptococci.

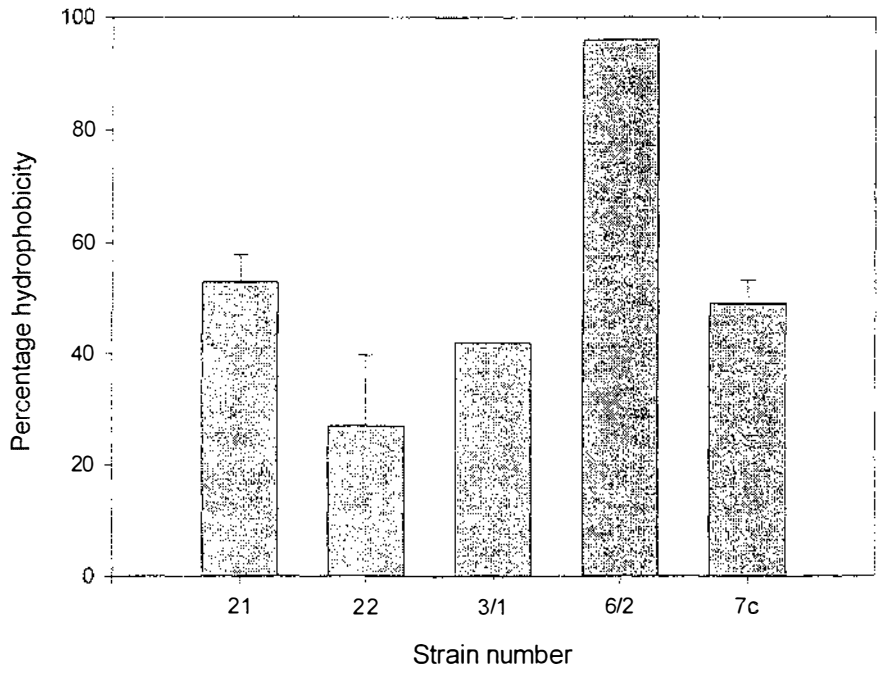


Figure 4.12 Hydrophobicity (mean and standard deviation from duplicate tests) of isolates of thermo-resistant streptococci from stainless steel exposed to pasteurised milk from manufacturing plants with no known problems (3/1, 6/2 and 7c) and reference cultures (21 and 22).

4.4 DISCUSSION

Thermo-resistant streptococci are present on the surface of dairy manufacturing plant post pasteurisation. They are the predominant microflora in the area, where temperatures are 37-40°C, and are not completely removed by standard cleaning procedures. Using a Robbins device designed specifically for sampling dairy manufacturing plant, the present trial highlighted the predominance of these bacteria. This data confirmed the observations of Hup *et al.* (1979) and Bouman *et al.* (1982). The number of bacteria colonising clean surfaces, installed in the manufacturing plant for 7 day periods, was similar over the 15 week sampling period. The low numbers (approximately 4.0 $\log_{10}$cells cm^{-2}) detected on the sample surfaces reflected the short run lengths that would limit the growth of viable cells. The proportion of viable cells in the biofilm was low (often < 2.0 $\log_{10}$cells cm^{-2}) and sporadic and this is most likely a reflection of the physiological state of the cells resulting from heat treatment during pasteurisation and cleaning procedures as well as from reduction of viability on storage before testing.

The discrepancy between cell numbers detected using epifluorescence microscopy (Figure 4.1) and conductance detection (Figures 4.2 and 4.3) occurred because the epifluorescence microscopy technique used detects both living and dead cells, whereas the Malthus detects only living cells. Therefore, techniques were used that allowed both the quantification of total cell numbers (living and dead) and the determination of cell viability. The results suggest an uneven distribution of viable cells on the surface of the pipe. As non-viable cells adhere as well as viable cells (Flint *et al.*, 1997, unpublished data), the large number of non-viable cells detected may have been due to either pasteurisation prior to adhesion or exposure to cleaning chemicals after adhesion. In the course of this work, it was determined that the refrigerated storage of samples prior to testing may result in loss of cell viability (data not shown). The variability in the number of viable cells is believed to be due

to some or all of these factors.

The high number of cells colonising the stainless steel samples taken at week 7 corresponded to a batch of milk containing a high number of bacteria, being pasteurised during this week.

The number of bacteria detected in the biofilms did not explain the variations in APC detected in the pasteurised milk (Figure 4.4). There are several possible reasons for this. Firstly, the proportion of viable cells in the biofilm may vary and this may not have been adequately determined due to the reduction in viability that occurs during the storage of the samples. Secondly, variations in the percentage of the area of plant colonised by these bacteria may affect the APC in the milk but may not be detected in the small (1 cm^2) sample zone. Thirdly, the APC measures all aerobic microflora, not only thermo-resistant streptococci that may originate from the biofilm. Finally, although many of the bacteria in the milk may have been released from the biofilm, the relatively small variations in the microflora detected in the milk (< 1 $\log_{10}$ ml) may have occured without any detectable difference in the amount of biofilm bacteria (*i.e.* within error).

The fact that cleaning did not result in a dramatic reduction in cell numbers suggests that standard caustic and acid cleaning regimes are ineffective in removing thermo-resistant streptococci from the surface. With the exception of week 7, the total number of bacteria associated with the coupons was consistently 3-4 $\log_{10}$cells cm^{-2}, suggesting that the number of bacteria on the surface was controlled by the operating conditions - in particular the short (6 h) length of the manufacturing runs which prevented the growth of viable cells to high numbers on the surface.

The high number of viable cells detected after cleaning in samples taken at week 4 cannot be explained by any known variation in the operation of the pasteuriser and may be due to a chance development of a thick biofilm at this sampling point. The

detection of viable bacteria on samples taken following cleaning is of concern, as these bacteria will enable further development of biofilm in later processing runs.

Further suggested investigations using the MRD in dairy manufacturing plant are to examine the effects of manufacturing run times, variations between manufacturing plants, seasonal variations and the accumulation of biofilm on stainless steel samples retained in the manufacturing plant for several weeks.

The dairy isolates examined in this study appeared morphologically (by Gram stain) and in colony morphology to be identical to other thermo-resistant streptococci. All were able to grow and clot milk at 45°C and therefore were similar to other thermo-resistant streptococci (Wood and Holzapfel, 1995).

The biochemical profiles of the isolates of thermo-resistant streptococci fell into two groups - one representative of *S. thermophilus* and another that differed from *S. thermophilus* or *S. bovis*. The biochemical profiles represented by types I, II and III (group 1) were similar to that of the *S. thermophilus* reference culture. The biochemical profiles (types IV and V) (group 2) of some of the isolates were more similar to that of *S. bovis* than to that of *S. thermophilus*. However, they were sufficiently different from both *S. thermophilus* and *S. bovis*, which are primarily associated with milk and cattle respectively, to suggest that they may belong to another species.

An examination of the fragments produced by restriction endonuclease analysis (REA), ribotying and RAPD analysis of this new species compared with *S. thermophilus* and *S. bovis* showed different patterns for each, supporting evidence for a separate species. REA has been used for many years to differentiate between different species but may also differentiate different isolates (*e.g.* serovars) within species (Marshall *et al.*, 1981). Ribotyping allows easier interpretation of differences between REA profiles as fewer bands are present. RAPD has been used to identify

many bacteria associated with the dairy industry including *Lactobacillus, Enterococcus,* and *Streptococcus* species (Cocconcelli *et al.* 1995). In the present trial, molecular techniques (REA, ribotyping and RAPD) used to screen the isolates clearly distinguished three different profiles corresponding to known isolates of *S. thermophilus*, *S. bovis* and another organism that also differed in its biochemical profile. This suggested that this unknown isolate belonged to a new species and that was provisionally named *S. waiu.*

The inability to produce a PCR product from some of the isolates from industry with primers specific for either *S. thermophilus* or *S. bovis* confirmed that some of these organisms were different from thermo-resistant streptococci most commonly associated with milk and dairy manufacture. Sequence analysis of the PCR product from amplification of a portion of genome encoding 16S rRNA showed that, although there was some similarity between sequence data for these isolates and those for *S. thermophilus, S. salivarius, S. bovis* and *S. equinus*, the unknown isolates formed a separate group. A specific primer selected from this sequence was successful in producing a PCR product from isolates that had so far been unable to be typed. Bentley *et al.* (1991) used comparative analysis of partial 16S rRNA sequences to categorise 24 *Streptococcus* species and found that *S. thermophilus, S. salivarius* and *S. vestibularis* formed one distinct group and that *S. bovis, S. equinus* and *S. alactolyticus* formed another distinct group. This is shown in the alignment of published 16S rRNA sequences presented in Figure 4.7. The alignment of the partial sequence of the unknown isolates from the present study, which have been named *S. waiu,* fits between these two groups. *S. waiu* appears to be most similar to the *S. bovis/equinus* group; however, it does possess some similarity to the *S. thermophilus/salivarius* group. This suggests that this organism is intermediate between the two groups. This is supported by the biochemical data (Table 4.1) where the *S. waiu* strains (types IV and V profiles) produced more positive reactions than *S. thermophilus* but fewer than *S. bovis.*

Strains represented by *S. bovis* and *S. equinus* have been found to be very heterogeneous (Farrow *et al.*, 1984). Genetic and biochemical studies led to the description of two new species - *S. alactolyticus* and *S. saccharolyticus* (Farrow *et al.*, 1984). However, *S. waiu* does not match the phenotypic or genotypic profiles for either of these species. There were twelve bp different in the sequenced region between *S. waiu* and the most closely related streptococcal species *S. equinus* for which 16S rRNA data are available. This compares with two bp different between the known species *S. equinus* and *S. bovis* and three bp different between *S. salivarius* and *S. thermophilus* in the same region.

In the present study, the phenotypic data and the 16S rRNA sequence suggest that the unknown isolates should be categorised as a new species. The unknown dairy isolates used in this study originated from different sources and were isolated at different times and it is therefore unlikely that they belong to a clone from an identical source.

DNA/DNA hybridisation confirmed the biochemical data that showed that *S. waiu* was more closely related to *S. bovis* than to *S. thermophilus*. The percentage homology values between *S. thermophilus, S. bovis* and *S. waiu* "3/1" (7.5-32.5%) were less than those reported for closely related thermo-resistant streptococci, *S. thermophilus* and *S. salivarius* (61-100%) (Farrow *et al.*, 1984), and therefore support the classification of *S. waiu* as a new species. In addition, the DNA/DNA homology recorded in these experiments was < 70%, which is regarded by the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics (Wayne *et al.*, 1987) as the minimum degree of relatedness expected for members of the same species.

The G + C ratio for *S. waiu* was 34.6% which was within the range of G + C values for streptococci (34-46%) (Hardie, 1986) but different from that for *S. thermophilus* or *S. bovis*.The G + C ratios of thermo-resistant streptococci of dairy origin, *S.*

thermophilus and *S. bovis*, are 40 and 36-39% respectively (Hardie, 1986). Although a difference in T_m < 5°C was recommended by the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics (Wayne *et al.*, 1987) for the classification of an organism as belonging to the same species, the difference in T_m between the species *S. thermophilus* and *S. bovis* based on the published G + C values is only 1.6°C. In the present study, the difference in T_m between *S. waiu* and *S. thermophilus* was 3°C. The standard deviation for the determination of the T_m has been reported as +/- 0.4°C (Marmur and Doty, 1962), and in the present trials was +/- 0.53°C.

The results from the biochemical profiles, PCR amplification of specific sequences encoding rRNA, comparisons between the partial sequences for 16S rRNA, FISH, REA and G + C ratios, support the classification of isolates 43, T, 7c, 6/2 and 3/1 into a new species with the proposed name *S. waiu*, which is the New Zealand Maori name for milk, reflecting the origin of these isolates.

The following summary describing this new species is proposed:

Description of *Streptococcus waiu* sp. nov.

Streptococcus waiu (waiu. New Zealand Maori n. milk). Cells are Gram-positive, non motile, non-sporeforming, cocci, 0.7 μm diameter in pairs or short chains. When the organism is grown on M17 agar at 37°C for 48 h, colonies are 0.5 mm dia. circular, convex, translucent and smooth. The organisms are homofermentative, facultatively anaerobic and catalase negative. The organisms will clot milk at 45°C and ferment galactose, D-glucose, D-fructose, D-mannose, N-acetyl glucosamine, salicin, cellobiose, maltose, lactose, melibiose, saccharose and D-raffinose. The DNA G + C content is 34.6 mol%. The habitat is milk and dairy products. The type strain is 3/1.

The discovery of this new species may assist in tracing the origin of thermo-resistant

streptococcus. The significance of this species in the contamination of dairy products needs to be evaluated.

All isolates originating from either biofilms or product from dairy manufacturing plants experiencing contamination of products with thermo-resistant streptococci were hydrophobic whereas the reference isolates and other isolates from milk were of mixed hydrophobicity. This suggests that isolates with the ability to form biofilms resulting in the contamination of dairy products are more hydrophobic than the general population. The higher hydrophobicity of isolates originating from manufacturing plants experiencing problems, compared with the hydrophobicity of bacteria isolated from the stainless steel surface during the monitoring trial, may reflect a difference in ability to colonise a surface. More extensive colonisation may have occurred in manufacturing plants experiencing problems with the contamination of products than in the manufacturing plant chosen for monitoring in this study. The possibility of extensive colonisation being associated with fouling with milk proteins was considered. However, this appears unlikely as other workers have shown colonisation with thermo-resistant streptococi occurs in parts of the manufacturing plant where protein fouling is low (Lehmann *et al.* 1990; Bouman *et al.*, 1982). In addition, the samples taken from dairy manufacturing plant for the present study showed no visible signs of protein fouling. The results from this study suggest that hydrophobicity is important in the development of a biofilm of thermo-resistant streptococci and that the contamination of dairy products with thermo-resistant streptococci is likely to result from biofilms.

These results differ from those of van der Mei *et al.* (1993), where eight strains of *S. thermophilus* isolated from heat exchanger plates in dairy manufacturing plants were all found to be relatively hydrophilic by the MATH test using hexadecane, rather than xylene, as the organic phase. The choice of organic phase may affect the results with the possibility of the hydrocarbon damaging the cell wall, resulting in measurements of the denatured surface (Denyer *et al.*, 1993). The zeta potentials of

the hydrocarbons used may also affect the results. This leads to the suggestion that the MATH test may not be a true indication of cell surface hydrophobicity (Busscher *et al.*, 1995a; van der Mei *et al.*, 1995).

The MATH test was chosen for this work as it is widely used and provides reproducible results. However, comparisons between different tests for hydrophobicity show a lack of correlation between methods unless the bacteria are highly hydrophobic or highly hydrophilic (Mozes and Rouxhet, 1987).

Hydrophobicity has been associated with the adhesion of bacteria to surfaces (Hood and Zottola, 1995). Hydrophobicity is believed to play an important role in the attachment of some streptococci to a substrate (Doyle *et al.*, 1990). Marín *et al.* (1997) reported a relationship between the strength of attachment of lactic acid bacteria to meat surfaces and the results from the MATH test using xylene ($r = 0.778$). However, Jameson *et al.* (1995) demonstrated that hydrophobicity (determined using hexadecane in the MATH test) did not affect the adhesion of *S. oralis* to salivary pellicle.

In the present work, the hydrophobicity determined by the MATH test using xylene as the organic phase is associated with the development of biofilms of thermo-resistant streptococci in dairy manufacturing plant

4.5 CONCLUSIONS

Thermo-resistant streptococci predominate on the surface of stainless steel in dairy manufacturing plant in the zone immediately following the pasteuriser where temperatures are ideal for the growth of these bacteria. This is the first report of a Robbins device, modified for installation in a dairy manufacturing plant, used to monitor the development of a biofilm in a dairy manufacturing plant. This is also the first report of conductance microbiology being used to determine the number of

viable bacteria colonising the surface of a dairy manufacturing plant.

S. thermophilus was the predominant species in the dairy manufacturing plants studied. However, a species that differs from all other species of thermo-resistant streptococci described, was isolated from both biofilm and milk. It is proposed to name this species *S. waiu,* waiu being the Maori name for milk.

All thermo-resistant streptococci isolated from either biofilms or milk from manufacturing plants where thermo-resistant streptococci are a problem were hydrophobic. It is likely that long operating times in a dairy manufacturing plant will allow the more hydrophobic isolates, selected through the ability to develop as biofilms, to be released and predominate in the product stream. The results from this study suggest that hydrophobicity is important in the development of biofilms of thermo-resistant streptococci and contaminated dairy products from dairy manufacturing plants are likley to originate from such biofilms.

Chapter 5

PROPERTIES OF THE CELL SURFACE OF THERMO-RESISTANT STREPTOCOCCI AFFECTING ADHESION TO STAINLESS STEEL

5.1 INTRODUCTION

Adhesion is one of the first steps in the development of a biofilm and is a complex process, involving passive and active mechanisms. The classical theories of adhesion suggest two to five stages in the process, involving passive van der Waals' attractive forces, electrostatic interactions, hydrophobic and steric forces and, later, more active adhesion through the production of polymers by the bacteria (Oliveira, 1992). Marshall *et al.* (1971) conveniently divided the adhesion process into two distinct phases - reversible and irreversible adhesion - relating to the force required to remove the cells from the surface. Busscher and Weerkamp (1987) proposed a three-step model, adding an extra initial stage where the cells are prevented from direct contact with the surface because of van der Waals' and electrostatic forces which are eventually overcome by stronger attractive forces. This stage, often referred to as the DLVO theory (developed independently by two research teams, Derjaguin and Landau (1941) and Verwey and Overbeek (1948), to explain the stability of colloids), stresses the influence of electrostatic interactions in the adherence of particles, including microorganisms, to solid surfaces (van Loosdrecht *et al.*, 1989). Characklis and Cooksey (1983) also expanded the two-stage model to include: cell transport to a wetted surface, adsorption of a conditioning film, adhesion of microbial cells, reactions within the biofilm and detachment of the biofilm. Whether all these stages are involved in the development of all biofilms is uncertain. For example, it is still unclear whether all microorganisms preferentially adhere to a conditioning film (Zottola and Sasahara, 1994).

In fact, the whole adhesion process is poorly understood. It is generally accepted that physico-chemical properties of both the substrate and bacterial surfaces have some role to play in the adhesion of bacteria (Carpentier and Cerf, 1993). These physico-chemical properties include electrostatic interactions, van der Waals' attractive forces,

hydrophobicity and steric forces. The importance of each varies depending on the type of microorganism, its stage of growth, the nature of the environment and the composition of the substrate. In addition, variations in the different test procedures used, *e.g.* for the cell surface hydrophobicity, make comparisons between the results from different laboratories difficult. The present state of knowledge suggests that the potential for microorganisms to adhere cannot be predicted from the physico-chemical properties of either the substrate or microbial surfaces (Carpentier and Cerf, 1993). The presence of bacterially produced components such as external appendages and extracellular polymers may also have an influence on the adhesion of some species (Oliveira, 1992).

It is generally agreed that the adhesion of microorganisms to surfaces is dependent on interactions between the bacterial species, the substrate and the environment and a better understanding of these factors will help in designing methods to control biofilms through the prevention of adhesion or by enhancing the removal of attached bacteria (Neu, 1996).

During preparation of a recent review on biofilms in dairy manufacturing plants (Flint *et al.,* 1997b), it became apparent that little is known about the factors involved in the adhesion of bacteria in this environment. Studies to date include those by Rönner *et al.* (1990) who studied the adhesion of spores of the *Bacillus* species (B. *cereus, B. licheniformis, B. polymyxa, B. subtilis* and *B. stearothermophilus*) often found in dairy manufacturing plants and concluded that *B. cereus* are the most adherent. The surface of *B. cereus* spores has a high relative hydrophobicity, a low zeta potential and morphological features such as long appendages - features that have all been postulated to aid adhesion to solid surfaces (Husmark and Rönner, 1992). Busscher *et al.* (1990) compared the abilities of two bacteria from the dairy industry, *Leuconostoc mesenteroides* and *Streptococcus thermophilus,* to adhere to solid surfaces and reported that *S. thermophilus* adheres more strongly than *L.*

mesenteroides to stainless steel surfaces.

The present study examined some of the properties of the cell surface of thermo-resistant streptococci (hydrophobicity, charge, polysaccharide and protein) and determined the role of each of these properties in the adhesion of thermo-resistant streptococci to stainless steel surfaces.

5.2 PROCEDURES

The isolates of thermo-resistant streptococci used in this study were obtained from the following sources.

(1) Commercially pasteurised milk (isolates 3/1, 6/2, 7c).

(2) Reference cultures - 21 (ATCC 19258) and 22 (NCTC 10353) (obtained from ESR Health, CDC Laboratories, Porirua, New Zealand).

(3) Dairy manufacturing plants - isolates H, T, EF_2, L3, L4 and BCD-7.

Full descriptions of these isolates are given in Section 3.1 and Chapter 4.

The number of cells adhering to stainless steel after different treatments was determined by epifluorescence microscopy (Section 3.4.1).

To determine the loss of viability on adhesion, thermo-resistant streptococci were inactivated by heat, ultraviolet (UV) light or formaldehyde (Section 3.6.2).

The relative surface charge of each isolate was tested by separation through anionic (Dowex AG 1 x 8 100-200 mesh) and cationic (Dowex AG 50W x 8 100-200 mesh)

exchange resins (Bio-Rad Laboratories Ltd, Glenfield, Auckland, New Zealand) (Section 3.6.3).

The hydrophobicity of the cell surface was determined by the microorganism adhesion to hydrocarbon (MATH) test (Section 3.5.10).

To investigate the effect of polysaccharides on adhesion, the amount of polysaccharide produced by each strain was compared with the numbers of cells adhering. The amount of extracellular polysaccharide (EPS) (expressed as μg dextran equivalent units 10^{-8} cells) produced by each strain was determined using the acid hydrolysis method of Dall and Herndon (1989), described by Evans *et al.* (1994) (Section 3.6.4). The effect of polysaccharides on adhesion was investigated further by comparing the number of cells of each strain adhering before and after treatment with one of three chemical treatments to disrupt cell surface polysaccharides (Gopal and Reilly, 1995) (Section 3.6.5). The effect of these chemical treatments in removing polysaccharide was determined by measuring the amount of polysaccharide in the supernatant fluid from treated cells using the acid hydrolysis method.

To enable investigations on the effect of proteins on adhesion, cells were treated with either sodium dodecyl sulphate (SDS) or trypsin to remove cell surface proteins (Gopal and Reilly, 1995) (Section 3.6.6). The effect of SDS on adhesion through the solubilisation of protein rather than the blocking of adhesion was determined by treating one strain with SDS for various times (2, 4, 6, 8, 10 and 20 min) and observing the effect on adhesion. To ensure that treatment with SDS or trypsin had no effect on the number of cells in suspension before adhesion, cells were counted before and after treatment (Section 3.4.4). The effect of removing cell surface proteins on hydrophobicity was determined by testing cells before and after treatment with trypsin (Section 3.5.10). To determine any changes in the morphology of the cell surface that occurred following treatment with trypsin, the cells were examined

by transmission electron microscopy (TEM) (Section 3.4.3). Polypeptides extracted from cells treated with either SDS or trypsin and from untreated cells were separated in a 10% polyacrylamide gel according to the method of Laemmli and Favre (1973) using a Hoefer Mighty Small minigel system (Biolab Scientific, Palmerston North, New Zealand) (Section 3.6.7). Polypeptides separated by SDS polyacrylamide gel electrophoresis (PAGE) were transferred to polyvinylidene difluoride (PVDF) membranes using a Hoefer transfer system (Section 3.6.8) for further analysis of the bands of interest. N-terminal sequencing of a polypeptide band of interest was carried out by Jo Mudford at the Department of Biochemistry and Biophysics, Massey University.

To release proteins attached to the cell wall, the cells were treated with enzymes (Section 3.6.6). This enabled the separation of proteins from the surface of *S. thermophilus* for analysis.

To visualise the location of cell surface proteins, antiserum to a polypeptide band of interest, cut from a PAGE gel, was prepared according to Section 3.6.10. The cells of strain "H" were immunolabelled (Section 3.6.12) and examined by TEM (Section 3.4.3).

To provide further evidence of the involvement of cell surface proteins in adhesion, the adhesion of cells of strain "H" treated with antiserum to the specific protein of interest was also determined (Section 3.6.11).

To investigate the effect of proteins from the environment on adhesion, *S. thermophilus* (H) was grown in a synthetic medium containing no peptones (Section 3.6.13), a trypsin digest of M17 medium and the permeate of M17 medium filtered through a 20 000 molecular weight (MW) cut-off membrane (Section 3.6.14).

To determine the effect of β-lactoglobulin on the adhesion of thermo-resistant streptococci, clean passivated stainless steel samples, 1 cm^2, were exposed to 0.3% β-lactoglobulin (Sigma Chemicals, Auckland, New Zealand) in sterile deionised water for 30 min before exposure to thermo-resistant streptococci (Section 3.6.15). The number of cells adhering was determined using the Malthus microbiological growth analyser. A mixture of thermo-resistant streptococci and 0.3% β-lactoglobulin was tested in the Malthus conductance growth analyser to ensure that β-lactoglobulin did not affect the viability of the cells.

Throughout this chapter, the relationship between the data for a particular factor and adhesion was determined using correlation coefficients (SigmaPlot, Jandel Corporation, San Rafael, California, USA).

5.3 RESULTS

5.3.1 The effect of cell viability on adhesion

The absence of growth on the surface of M17 plates, from a 10^{-2} inoculum of washed cells from an overnight culture resuspended in sterile dionised water, treated with heat, UV light or formaldehyde, confirmed that the cells were no longer viable (Table 5.1). However, the number of cells attaching to the stainless steel, estimated by epifluorescence microscopy, was similar for both treated and untreated samples at 4.9-5.3 log_{10}cells cm^{-2} (Table 5.1). The lowest number of cells attaching to the stainless steel occurred with samples exposed to formaldehyde.

5.3.2 The effect of cell surface charge on adhesion

All 11 isolates tested possessed a net negative surface charge with a greater retention of cells on the anionic than the cationic exchange resin (Figure 5.1). The percentage

of cells with a net positive charge ranged from 5 to 97% and the percentage with a net negative charge was 20-99% (the mean standard deviation (SD) for both tests was 0.79). There was no relationship between the percentage of positively or negatively charged cells and the number of cells adhering to stainless steel coupons ($r^2 = 0.13$ and 0.25 respectively) (Figures 5.2 and 5.3). For strain H, similar numbers of cells were retained on both anionic and cationic exchange resins, presumably due to an equal distribution of positive and negative charges on the cell surface. For any strain, a proportion of cells no charge would not be retained by either resin.

Table 5.1

The effect of viability of *S. thermophilus* (H) on adhesion

Treatment	Colonies on M17 agar (log_{10}cfu ml^{-1})	Adhered cells (log_{10}cells cm^{-2})
Untreated	7.08	5.3 +/- 0.2
100°C 10 min	< 2.0	5.3 +/- 0.06
0.04% Formaldehyde	< 2.0	4.9 +/- 0.05
UV 10 min	< 2.0	5.1 +/- 0.3

5.3.3 The effect of cell surface hydrophobicity on adhesion

The percentage of hydrophobic cells ranged from 22 to 99% (SD 1.4) (Chapter 4). No relationship could be established between the degree of hydrophobicity and the adhesion of cells to stainless steel ($r^2 = 0.09$) (Figure 5.4).

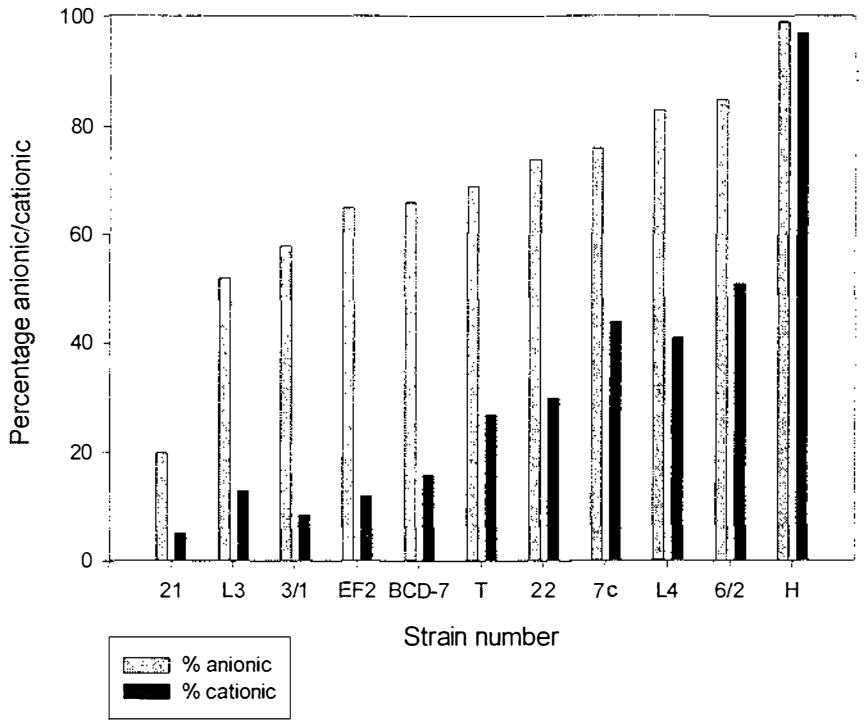


Figure 5.1 Percentage of cells of 11 strains of thermo-resistant streptococci retained on anionic and cationic exchange resins (SD 0.79).

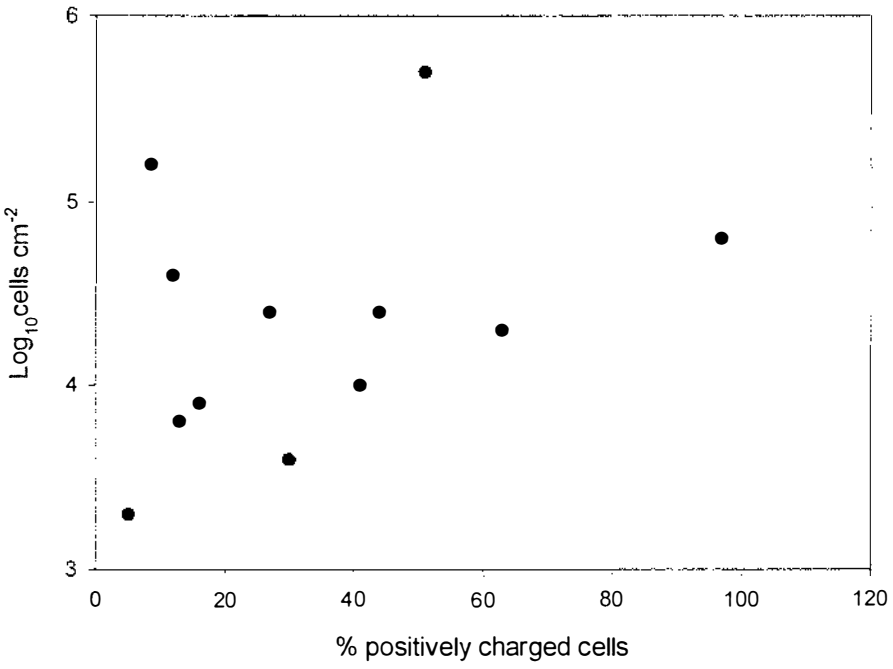


Figure 5.2 Relationship between cell surface positive charge and adhesion. based on the percentage of cells binding to anionic exchange resin.

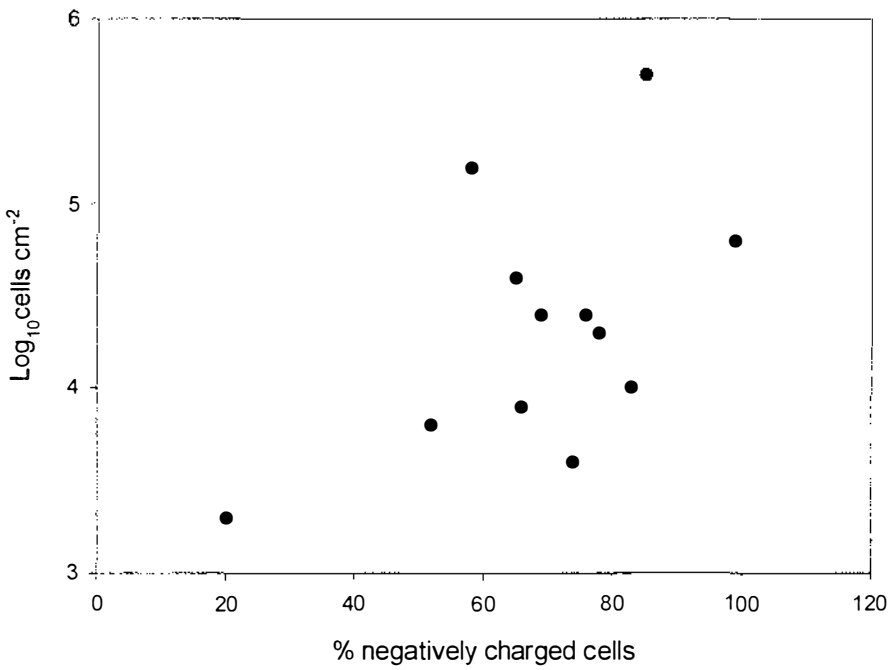


Figure 5.3 Relationship between cell surface negative charge and adhesion. based on the percentge of cells binding to cationic exchange resin.

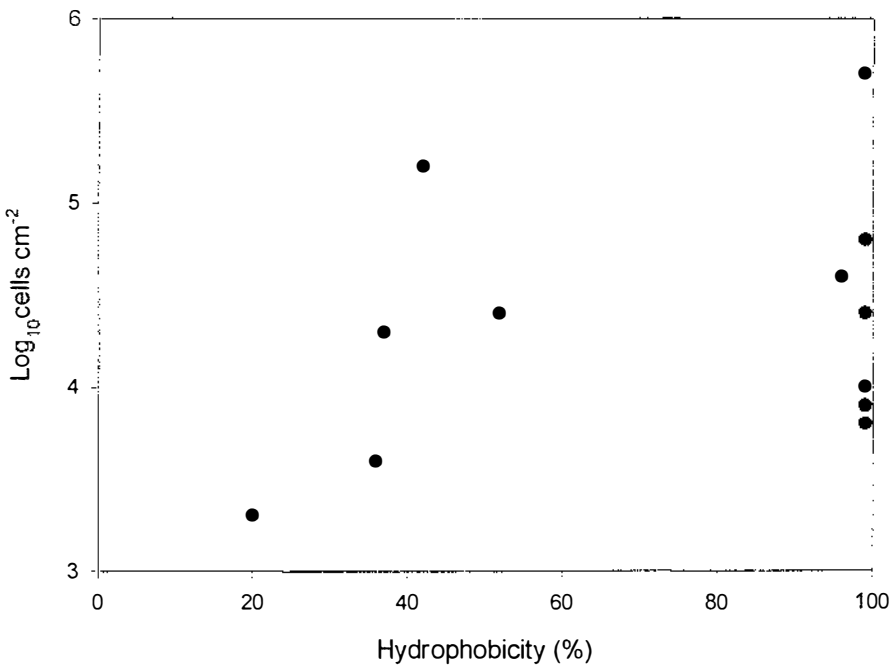


Figure 5.4 Relationship between cell surface hydrophobicity and adhesion.

5.3.4 The effect of EPS on adhesion

The amount of EPS recovered from standardized cell suspensions varied between 0.17 and 1.65 μg dextran equivalent units 10^{-8} cells (Figure 5.5). The amount of EPS could not be related to the source of the strains or the species and there was no relationship between the amount of EPS and the number of cells attaching to stainless steel after 30 min exposure (r^2 = 0.25) (Figure 5.6).

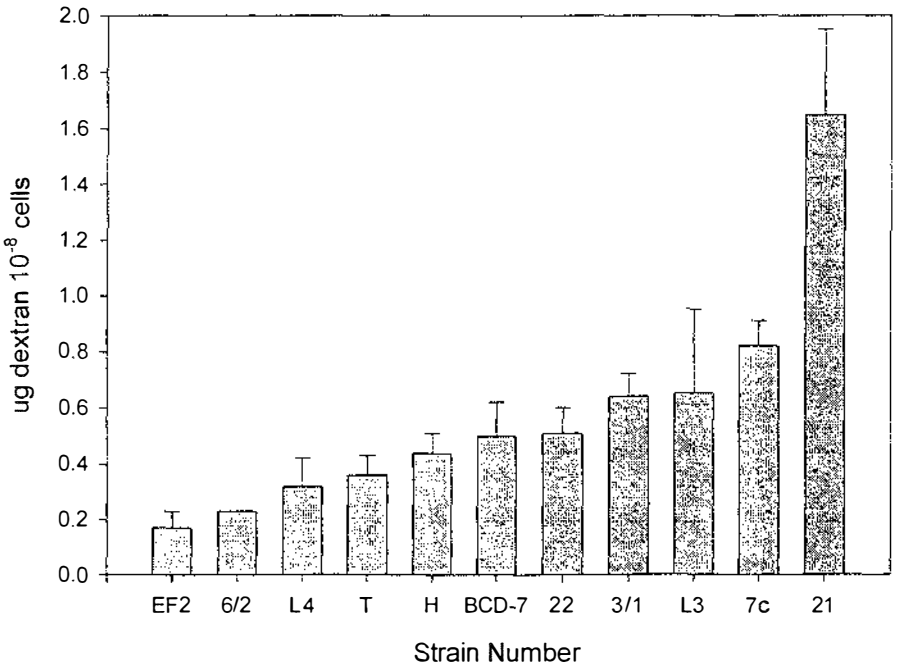


Figure 5.5 EPS produced by 11 strains of thermo-resistant streptococci (mean and standard deviation from duplicate tests).

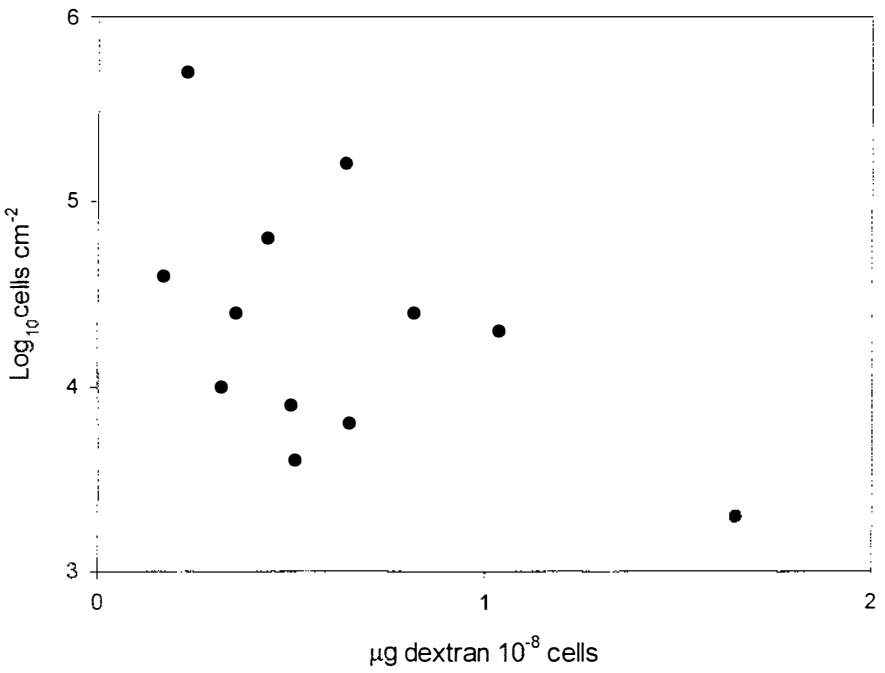


Figure 5.6 Relationship between EPS and adhesion of thermo-resistant streptococci to stainless steel.

5.3.5 Adhesion following treatment to disrupt the polysaccharide components of the cell walls

Treatments to disrupt EPS (lysozyme, sodium metaperiodate or trichloroacetic acid (TCA)) produced no net increase or decrease in the number of cells adhering to stainless steel (Figures 5.7, 5.8 and 5.9). After treatment with lysozyme, four strains showed increased adhesion and seven showed decreased adhesion. Likewise, after treatment with sodium metaperiodate and TCA, three and six strains respectively showed increased adhesion whereas eight and four strains respectively showed decreased adhesion. One strain showed no change in the number of cells adhering after treatment with TCA.

In some instances, treatments to disrupt EPS produced large changes in the number of cells of particular strains adhering. For example, there was a 99.9% reduction in the number of cells of strain L4 adhering following treatment with sodium metaperiodate. Conversely there was a 97% increase in the number of cells of strain 6/2 adhering following treatment with TCA.

To confirm the effect of treatments to disrupt the polysaccharides of the cells, the supernatants from treated cells were tested for polysaccharide. The mean amount of EPS in the supernatant following the treatment of cells with lysozyme and sodium metaperiodate was 1.14 and 0.58 dextran equivalent units 10^{-8} cells respectively, compared with 0.18 dextran equivalent units 10^{-8} cells for the untreated cells. The increase in polymer recovered after lysozyme or sodium metaperiodate treatment was due to the fact that these treatments, by disrupting the cell wall polysaccharides, enhanced their removal in the subsequent sonication step of the polymer assay. The amount of EPS recovered from cells treated with TCA (0.16 dextran equivalent units 10^{-8} cells) was similar to that from the untreated cells. The removal of EPS from the

cells by sonication following treatment with lysozyme did not affect the adhesion of the cells to stainless steel (6.3 $\log_{10}$ cells cm^2 before sonication and 6.5 $\log_{10}$cells cm^{-2} after sonication).

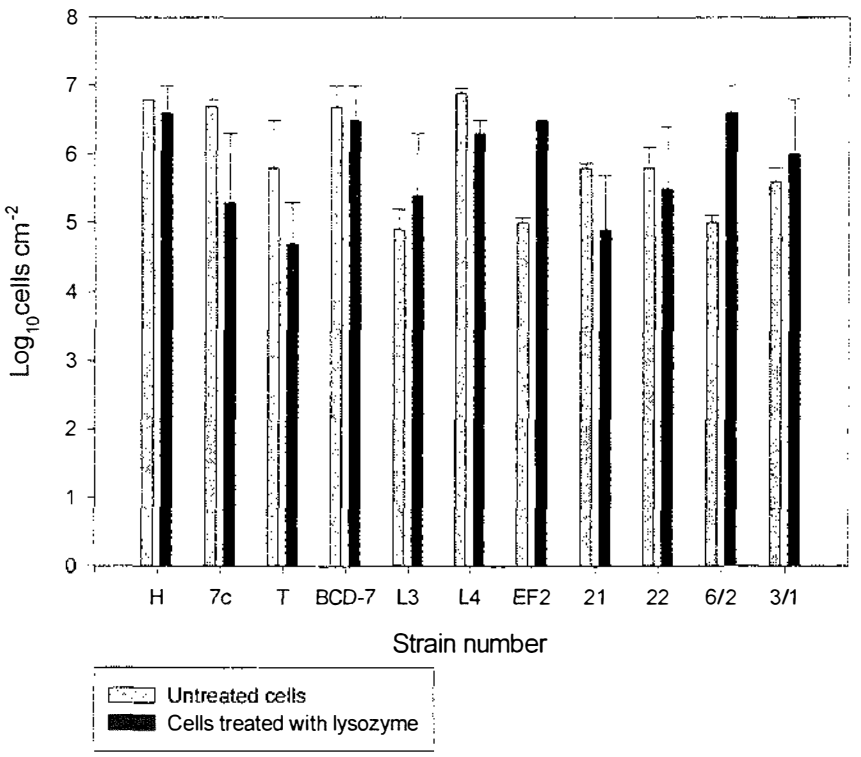


Figure 5.7 Adhesion of 11 strains of thermo-resistant streptococci following treatment with lysozyme (mean and standard deviation from duplicate tests).

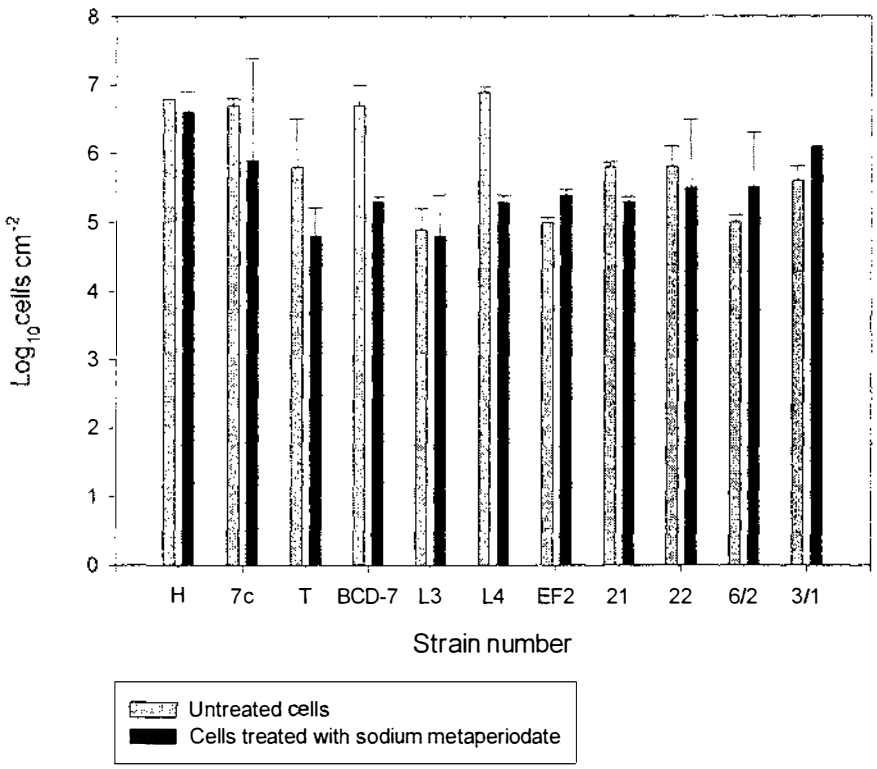


Figure 5.8 Adhesion of 11 strains of thermo-resistant streptococci following treatment with sodium metaperiodate (mean and standard deviation from duplicate tests).

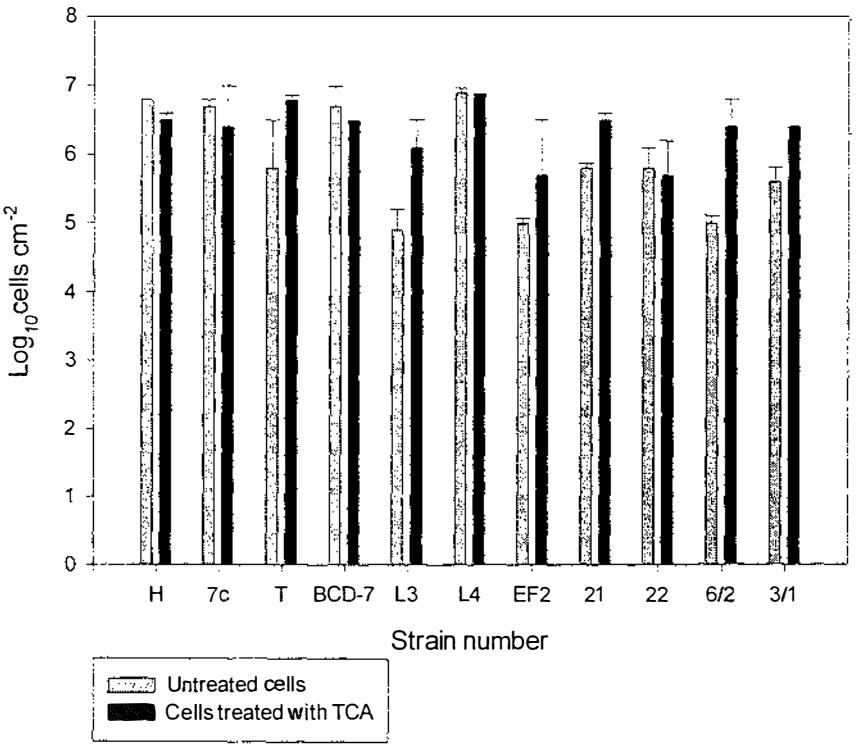


Figure 5.9 Adhesion of 11 strains of thermo-resistant streptococci following treatment with TCA (mean and standard deviation from duplicate tests)

5.3.6 Adhesion following treatment to remove proteins

Treatment of standardised cell suspensions with either SDS or trypsin reduced the number of cells attaching to stainless steel (Figures 5.10 and 5.11). For all 11 strains of thermo-resistant streptococci tested, mean reductions of 2.0 $\log_{10}$cells cm^{-2} (SD 0.98) and 2.0 $\log_{10}$cells cm^{-2} (SD 0.88) in the number of cells adhering occurred following treatment with trypsin and SDS respectively. The reduction in adhesion for individual strains following either treatment was similar.

The progressive effect of SDS on the adhesion of cells suggested that solubilisation of protein rather than blocking (expected to occur instantaneously) was responsible for the reduction in adhesion. Treatment with SDS over shorter times (2, 4, 6, 8, 10 and 20 min) produced a progressive reduction in the number of cells attaching to stainless steel, reaching a maximum 100-fold reduction after treatment for 6 min (Figure 5.12). The possibility of a progressive blocking activity cannot be fully discounted using these results in isolation. However treatment was assoicated with the loss of cell proteins visualised on SDS-PAGE (see later).

SDS and trypsin had negligible effect on the number of cells in suspension. For the 11 strains before treatment, the mean number of total cells in suspension was 8.3 $\log_{10}$cells ml^{-1} (SD 0.1) and following SDS and trypsin treatment reduced to 8.0 $\log_{10}$cells ml^{-1} (SD 0.4) and 8.1 $\log_{10}$cells ml^{-1} (SD 0.4) respectively.

Treatment with trypsin reduced the hydrophobicity of *S. thermophilus* (H) from 98.7 +/- 0.26% to 52.4 +/- 7.2%. Although hydrophobicity does not appear to be important in the adhesion of thermo-resistant streptococci, the predominance of hydrophobic isolates in dairy manufacturing plant suggests that hydrophobicity has some role to play in the development of biofilms (Chapter 4).

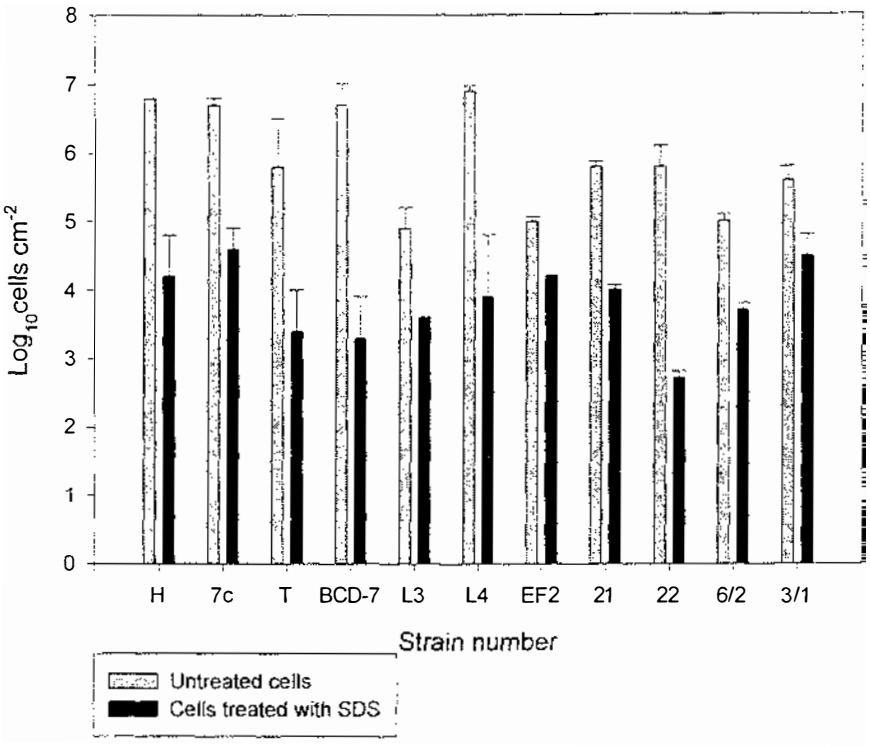


Figure 5.10 Adhesion following treatment with SDS (mean and standard deviation from duplicate tests).

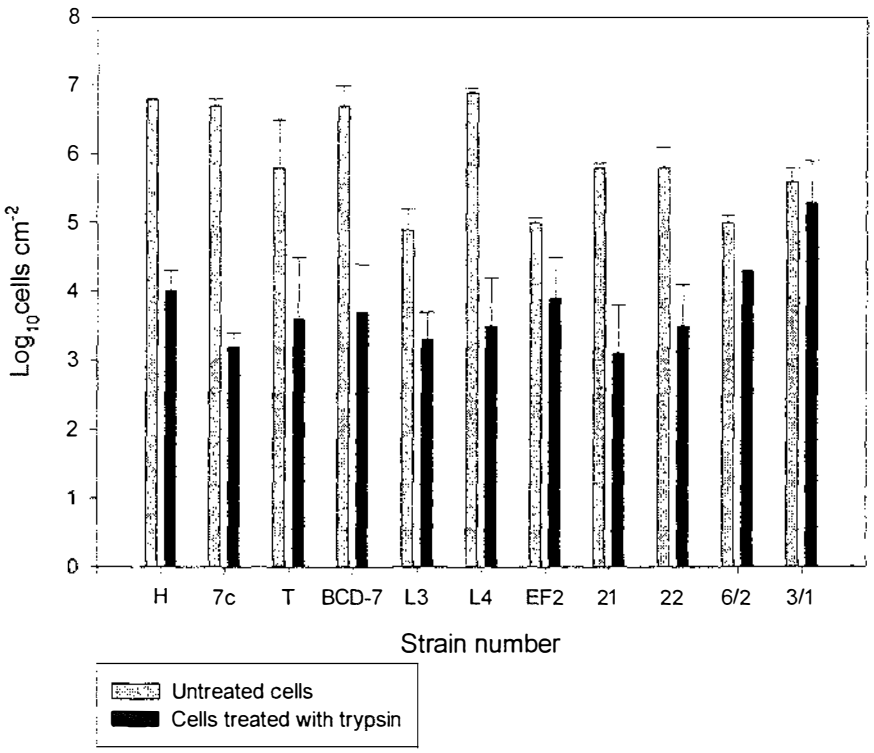


Figure 5.11 Adhesion following treatment with trypsin (mean and standard deviation from duplicate tests).

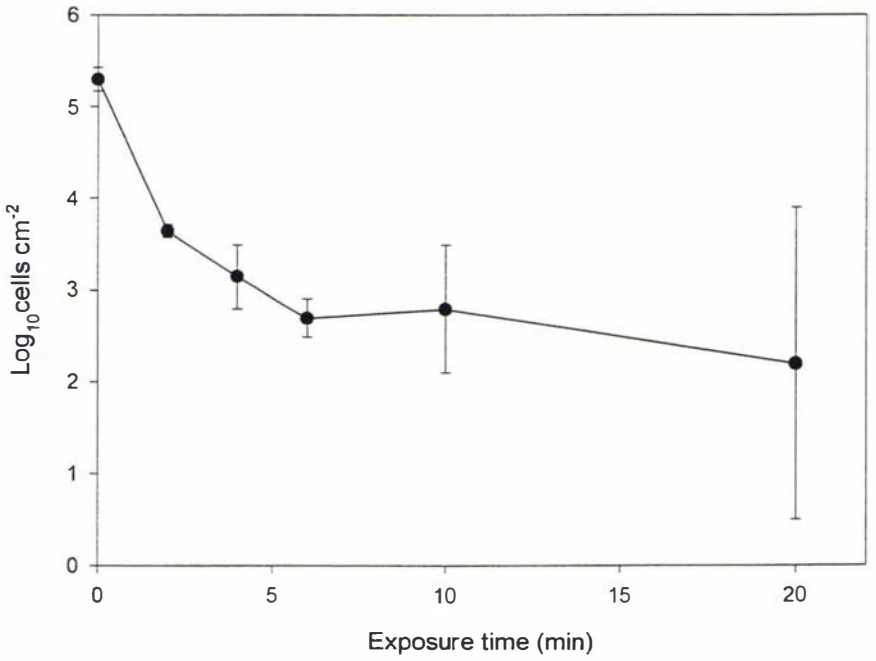


Figure 5.12 Adhesion following SDS treatment of samples of cells over 20 min (mean and standard deviation from replicate tests).

5.3.7 TEM of cells before and after treatment with trypsin

Differences between untreated and trypsin treated cells were seen using TEM of sectioned preparations of resin-embedded cells (Figures 5.13 and 5.14). The distance between the cell wall and the cell membrane appeared to be 1.5-2 times greater in the trypsin treated cells than the untreated cells. This observation may also be interpreted as a swelling of the cell wall. Negative staining did not reveal any surface features (*e.g.* fimbriae) that may have been involved in adhesion.

5.3.8 SDS-PAGE of total cell proteins

SDS-PAGE of the cell proteins before and after treatment with SDS showed a reduction in one polypeptide of approximately 55 kDa following treatment with SDS (Figure 5.15). Treatment over 2-20 min with SDS resulted in a loss of the 55 kDa band after 6 min and this corresponded to the observed effect of SDS on the number of cells adhering when the treated cell suspension was exposed to stainless steel (Figure 5.12). Treatment with trypsin resulted in the absence of any polypeptides between 12 and 67 kDa.

5.3.9 N-terminal sequencing of the "adhesion protein"

The N-terminal sequence of the 55 kDa polypeptide associated with adhesion was:

RIVTQGMXWH (arginine, isoleucine, valine, threonine, glutamine, glycine, methionine, unknown, tryptophan, histidine).

This most closely resembles the sequence for β-lactoglobulin.

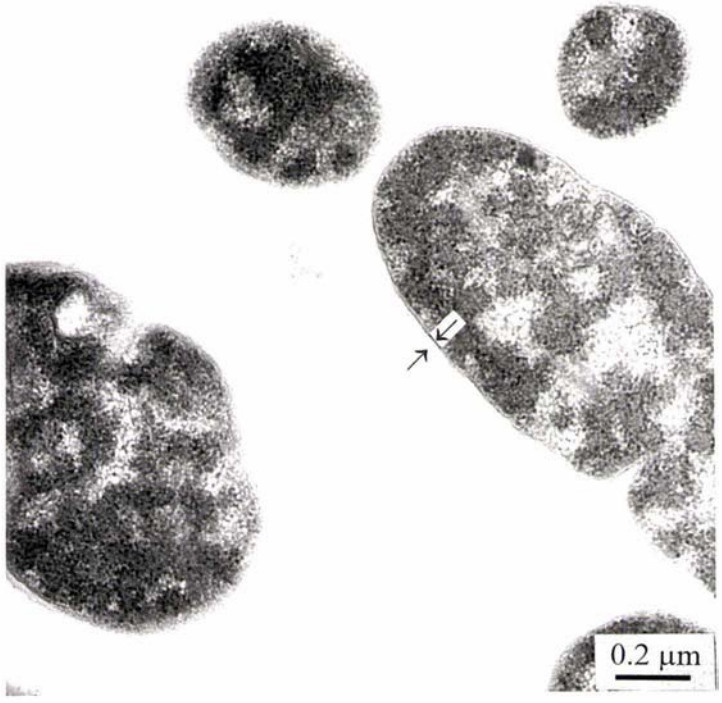


Figure 5.13 TEM of *S. thermophilus* (H) before treatment with trypsin, 49 000 x magnification.

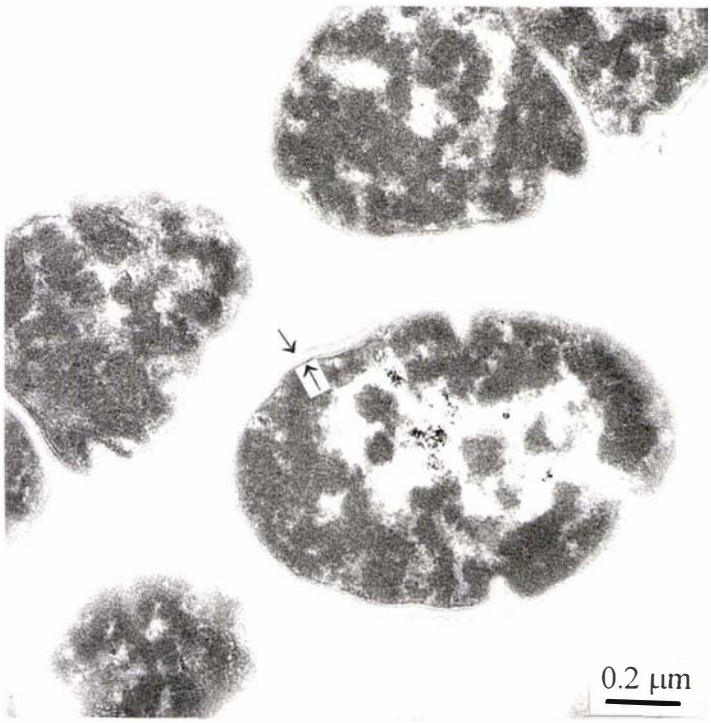
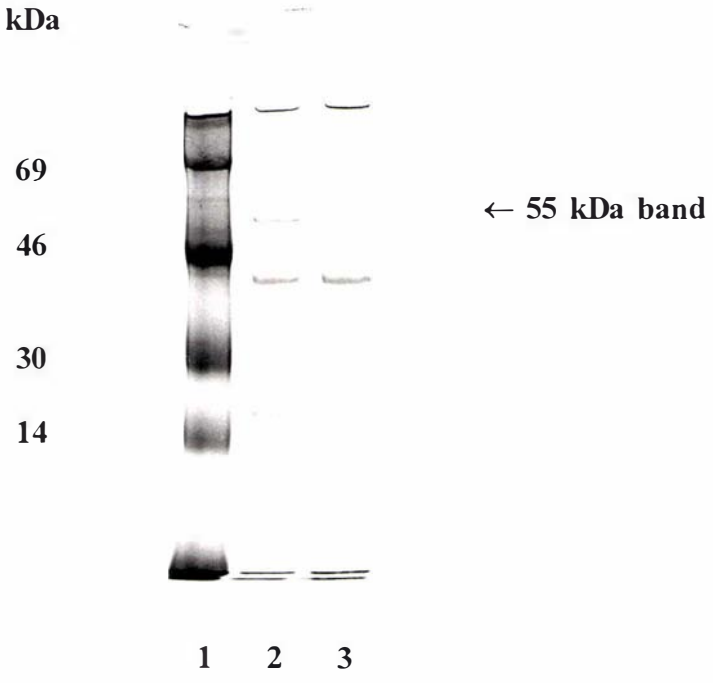


Figure 5.14 TEM of *S. thermophilus* (H) following treatment with trypsin, 49 000 x magnification.



1 = MW standard

2 = Total cell proteins - untreated cells

3 = Total cell proteins - SDS-treated cells

Figure 5.15 SDS-PAGE of total cell proteins from *S. thermophilus* (H) before and after treatment of the cells with SDS.

5.3.10 Extract of the cell wall of *S. thermophilus* (H)

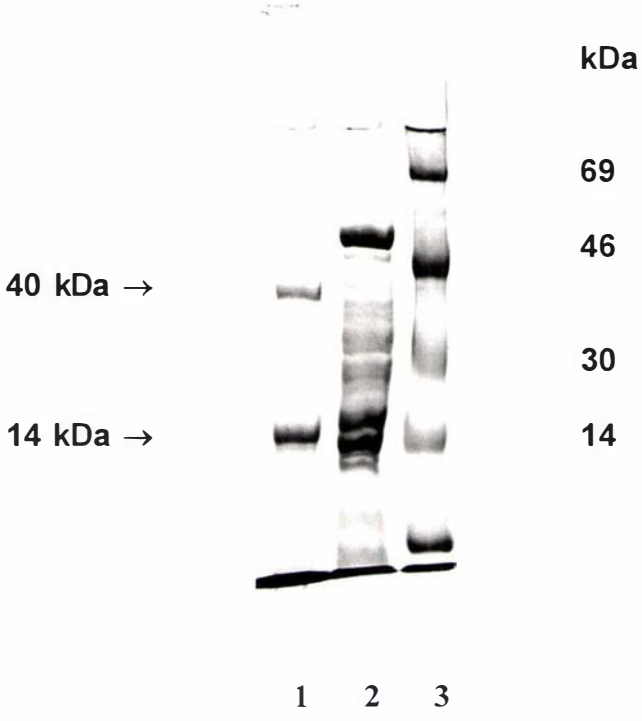
Two polypeptides (14 and 40 kDa) were identified in SDS-PAGE separation of the extract from the cell wall of *S. thermophilus* (H) (Figure 5.16).

5.3.11 Antiserum to the "adhesion protein"

Antiserum produced against the adhesion protein, bound most strongly to the region represented by the 55kDa protein when antiserum was used to probe a Western blot of total cell proteins separated by SDS-PAGE. There was some weaker cross-reactions with some other cell proteins.The antisera also bound to one of the two polypeptides extracted from the cell wall (Figure 5.17).

5.3.12 Inhibition of adhesion following treatment with antisera to the "adhesion protein"

The number of *S. thermophilus* (H) cells adhering to stainless steel following exposure to antibodies to the adhesion protein was reduced by approximately 100-fold (6.5 +/- 0.2 $\log_{10}$cells cm^{-2} before treatment with antisera, and 4.2 +/- 0.35 $\log_{10}$cells cm^{-2} after treatment with antisera). No reduction in the number adhering was seen following exposure to normal rabbit serum (Figures 5.18, 5.19 and 5.20).

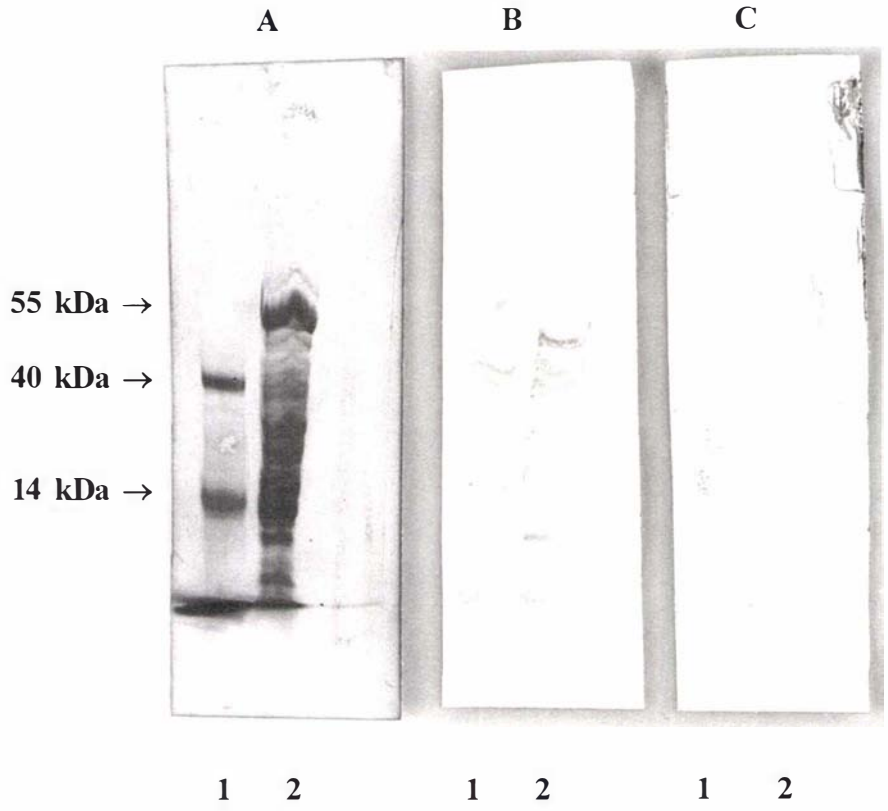


1 = Cell wall extract

2 = Total cell proteins

3 = MW standard

Figure 5.16 SDS-PAGE of total cell proteins and a cell wall extract from *S. thermophilus* (H).



1 = Cell wall extract

2 = Total cell proteins

Treated with:

A = Coomassie blue stain

B = Antiserum to adhesion protein, anti-rabbit peroxidase conjugate and substrate

C = Normal rabbit serum, anti-rabbit peroxidase conjugate and substrate

Figure 5.17 Western blots of total cell proteins and a cell wall extract from *S. thermophilus* (H). Antisera to the adhesion protein (B) binds most strongly to the region represented by the 55 kDa polypeptide, associated with adhesion.

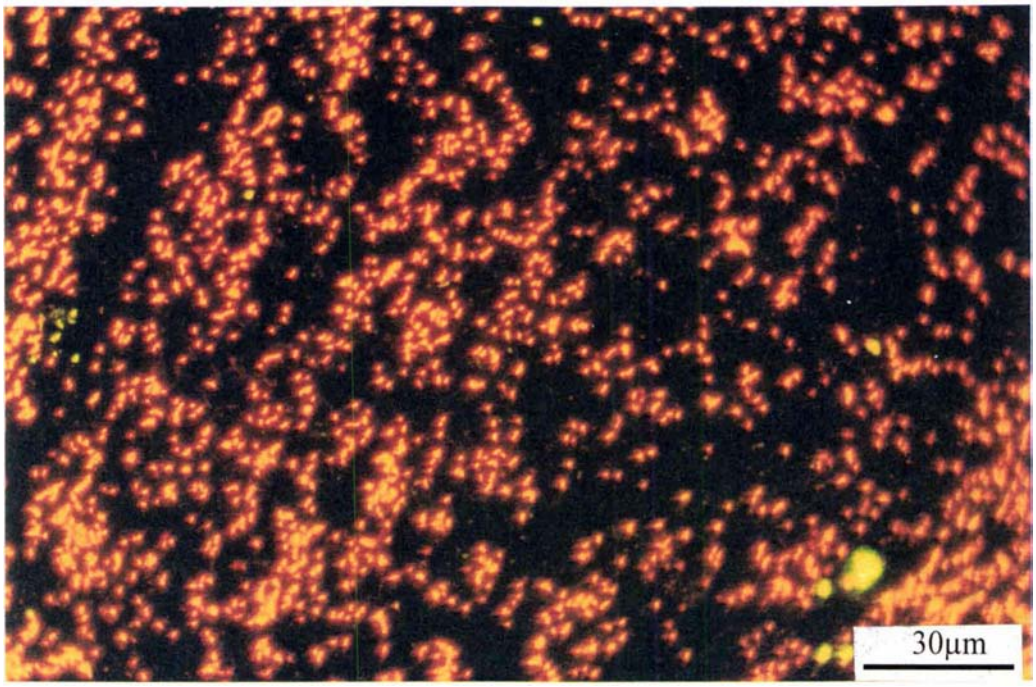


Figure 5.18 *S. thermophilus* (H), not treated with antiserum, adhered to stainless steel. Acridine-orange-stained cells, 200 x magnification.

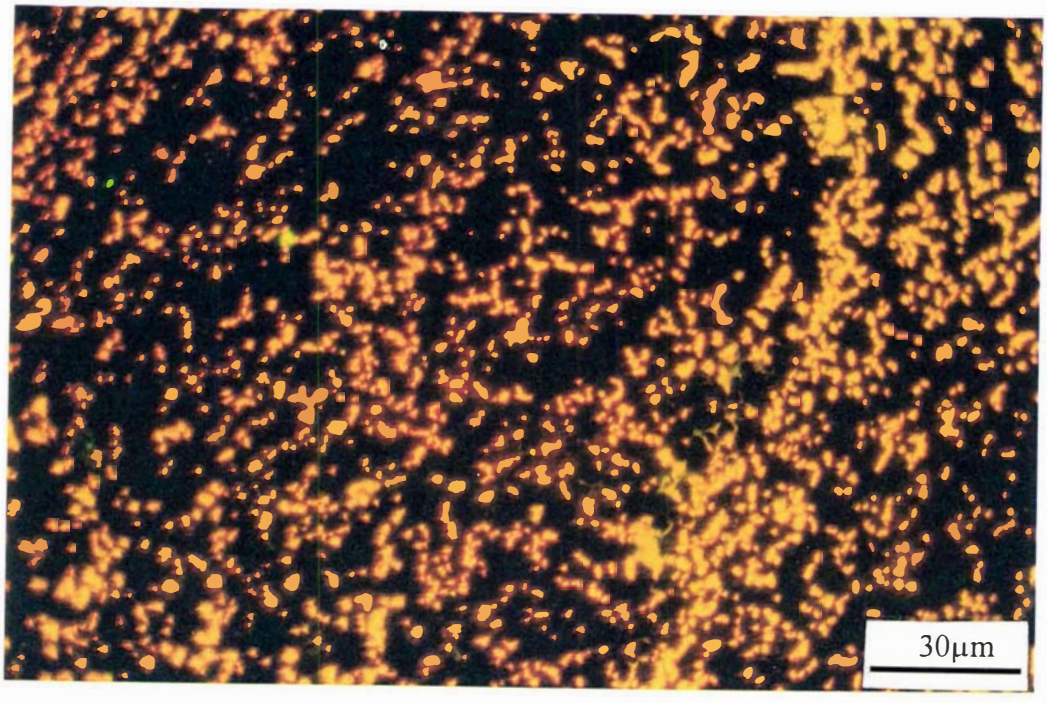


Figure 5.19 *S. thermophilus* (H), treated with normal rabbit serum, adhered to stainless steel. Acridine-orange-stained cells, 200 x magnification.

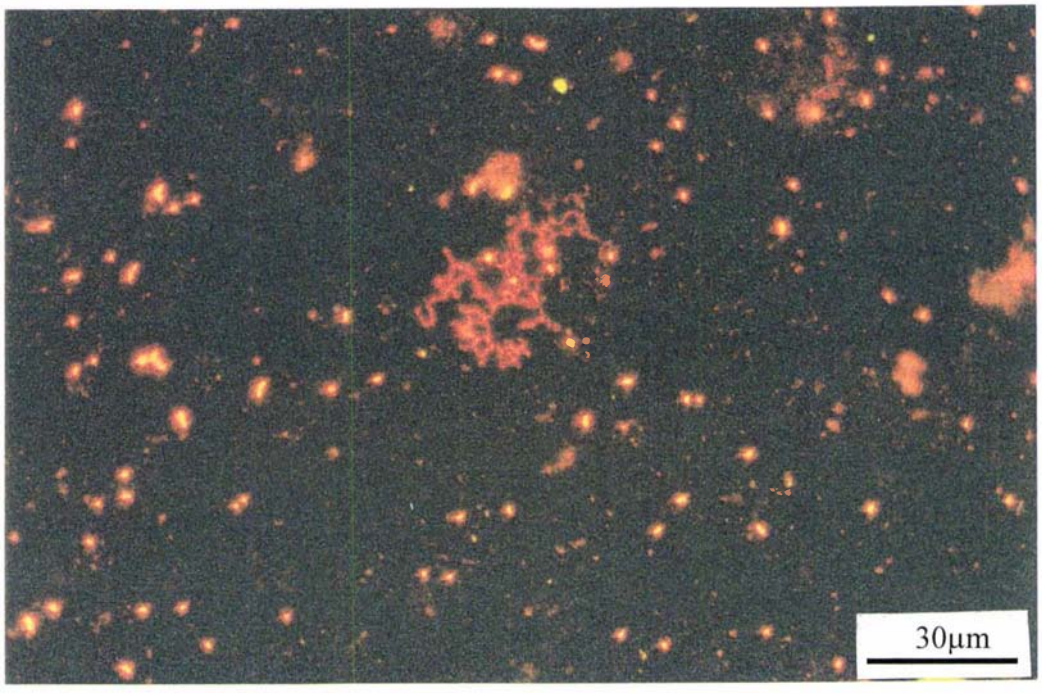


Figure 5.20 *S. thermophilus* (H), treated with rabbit antiserum to the "adhesion protein", adhered to stainless steel. Acridine-orange-stained cells, 200 x magnification.

5.3.13 Localisation of the "adhesion protein" on the cell surface using immuno-gold-labelled conjugate and TEM

To demonstrate the presence of the "adhesion protein" on the surface of the cells, *S. thermophilus* cells were immunolabelled using specific antiserum to the adhesion protein. Cells that were pre-exposed to antibodies to the adhesion protein, followed by gold- labelled anti-rabbit conjugate and thoroughly washed, were observed to have changed colour, indicating the binding of the gold conjugate. No colour change was present on cells exposed to normal rabbit serum and the gold conjugate. Under TEM, gold particles were observed on the surface of the cells that had been exposed to the specific antiserum (Figure 5.21) and were not present on cells exposed to normal rabbit serum. No structures that may be associated with these proteins were observed.

5.3.14 Adhesion following growth in synthetic and modified media

To demonstrate the possibility of the adhesion protein being derived from the environment, *S. thermophilus* was grown through 10 passages in synthetic medium containing no peptones, a trypsin digest of M17 medium, or permeate from the ultrafiltration of M17 medium. Cells grown in any one of these media were less able to adhere to stainless steel (Figure 5.22). However, cells produced in synthetic medium still contained the 55 kDa adhesion protein (Figure 5.23).

5.3.15 The effect of exposing stainless steel to β-lactoglobulin on the adhesion of thermo-resistant streptococci

Further evidence of the likely role of β-lactoglobulin or a complex of β-lactoglobulin being involved in the adhesion of thermo-resistant streptococci was obtained by monitoring adhesion of *S. thermophilus* to stainless steel that had been exposed to β-lactoglobulin. The number of bacteria adhering to stainless steel pre-exposed to β-lactoglobulin was 0.5 +/- 0.5 $\log_{10}$cells cm^{-2} compared with 3.7 +/- 0.2 $\log_{10}$cells cm^{-2} for adhesion to stainless steel that had not been exposed to β-lactoglobulin.

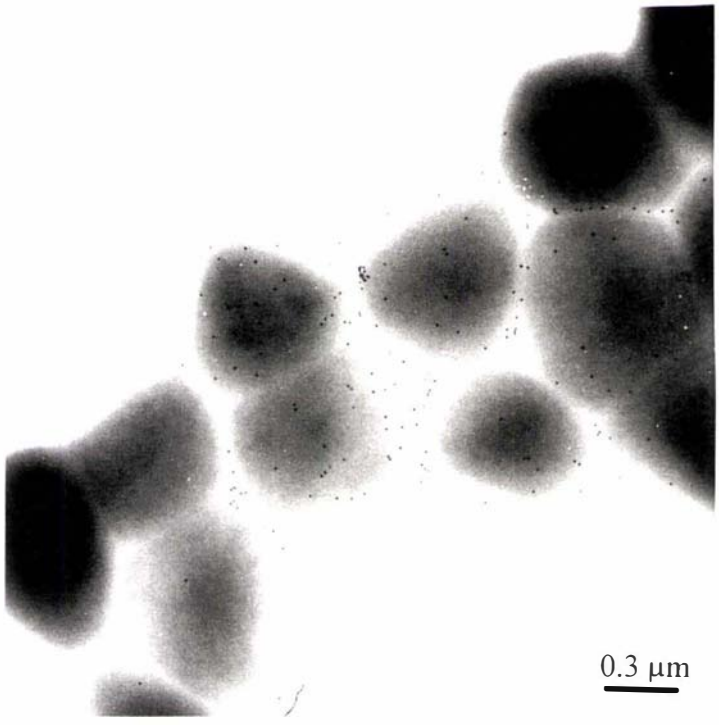
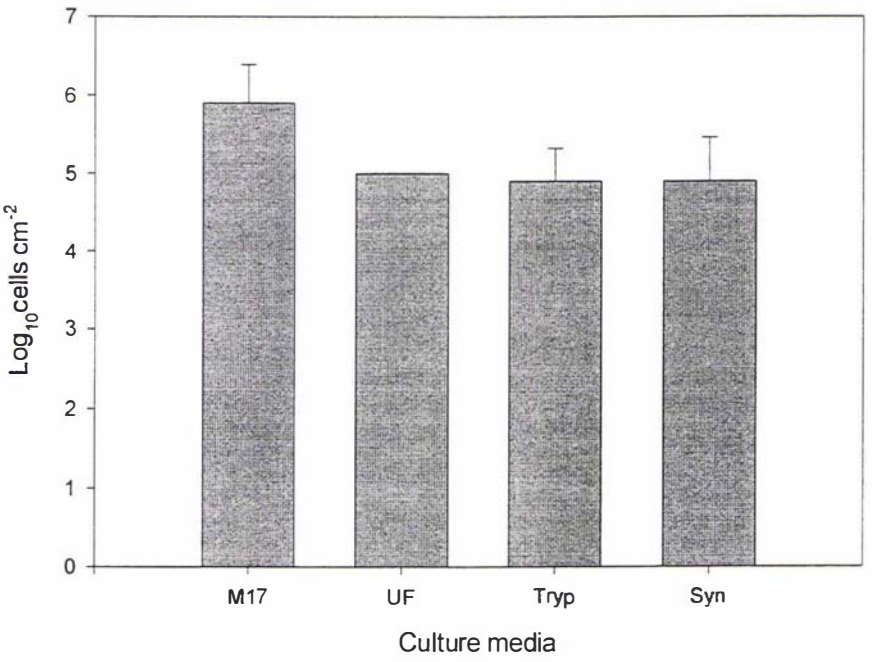


Figure 5.21 TEM of immuno-gold-labelled cells of *S. thermophilus* treated with antiserum to the adhesion protein, 31 000 x magnification. (The small black dots represent the gold-labelled conjugate bound to the specific antibodies reacting with the adhesion protein on the surface of the cell. The cells were not stained, therefore they appear as diffuse shadows)



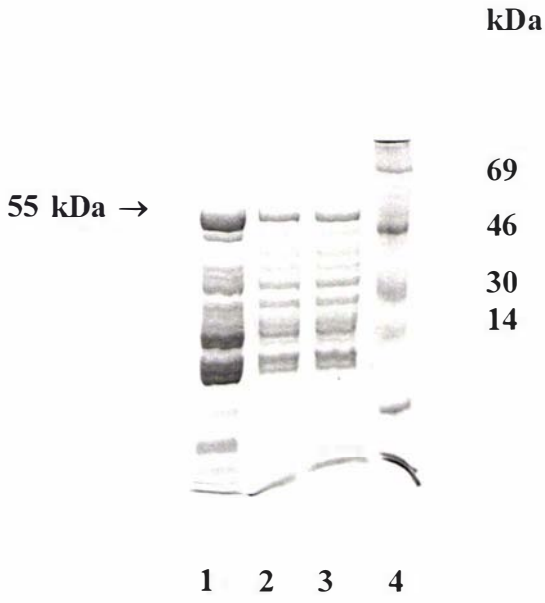
M17 = Peptone based growth medium

UF = Ultrafiltered (20 000 MW) M17 medium

Tryp = Trypsin digest of M17 medium

Syn = Synthetic medium

Figure 5.22 Adhesion of thermo-resistant streptococci grown in synthetic or modified media (mean and standard deviation from duplicate tests).



Total cell proteins from *S. thermophilus* grown in:

1 = M17 medium
2 = Synthetic medium
3 = Synthetic medium
4 = MW standard

Figure 5.23 SDS-PAGE of thermo-resistant streptococci grown in synthetic medium.

5.4 DISCUSSION

Factors affecting the adhesion of thermo-resistant streptococci to stainless steel were determined in order to learn more about the development of biofilms of thermo-resistant streptococci (studied in Chapter 4) that persist in dairy manufacturing plant.

The effect of cell viability on adhesion was determined. As cells of *S. thermophilus* that had been killed by heat, UV light or formaldehyde adhered with almost the same frequency as viable cells, it appears that the adhesion of thermo-resistant streptococci to stainless steel surfaces does not require physiologically active cells. This is in contrast to studies with other organisms in the dairy industry, where the adhesion of *Pseudomonas fluorescens* was reduced by up to 99% following the inactivation of cells with heat (Czechowski, 1990). In the present trials, the small reduction in the numbers of *S. thermophilus* adhering following treatment with formaldehyde may have resulted from alterations to the cell surface proteins. As similar numbers of inactivated and viable thermo-resistant streptococci adhered to stainless steel, conditions that were lethal to the bacteria were able to be used in studies to determine the mechanisms of adhesion.

Cell surface charge, hydrophobicity and polysaccharide production were investigated to determine their role in the adhesion of thermo-resistant streptococci to stainless steel. All these factors have been reported to be involved in the adhesion of bacteria to surfaces with the importance of these factors varying from species to species (Marshall *et al.,* 1971; Doyle *et al.,* 1990; Neu, 1992; Hood and Zottola, 1995). In this trial, no relationship was shown between adhesion and any of these three factors. This suggests that other factors are of greater importance in controlling this first stage in biofilm development.

Although it was not possible to demonstrate a relationship between the proportion of negatively charged cells and adhesion, surface charge may still influence the adhesion process. The predominantly negative charge on the bacterial surface is likely to cause repulsion of bacteria from surfaces that are negatively charged. The studies of van der Mei *et al.* (1993), using microelectrophoresis to assess zeta potentials, concluded that thermo-resistant dairy streptococci were only slightly negatively charged; therefore this negative electrostatic interaction with surfaces may be low. The present study, agreed with van der Mei *et al.*(1993) in that the cell were predominantly negatively charged, however the method used was unable to accurately determine the magnitude of that charge.

In the present trials, the hydrophobicity of the individual thermo-resistant streptococci used differed; however, there was no obvious relationship with the adhesion of cells to a stainless steel substrate. This result was similar to those of Jameson *et al.* (1995) who demonstrated that hydrophobicity and surface proteins did not affect the adhesion of *Streptococcus oralis* to salivary pellicle.

EPS are believed to be important in the adhesion of some bacteria to surfaces and have been termed "adhesive polymers" (Neu, 1992). Marshall *et al.* (1971) suggested that EPS plays a role in both the initial adhesion and the irreversible adhesion. Some authors consider that EPS can promote a preconditioning of the surface, making adhesion more favourable (Oliveira *et al.*, 1994). Herald and Zottola (1989) showed that compounds that bind to or disrupt carbohydrates, such as sodium metaperiodate, Cetavlon and concanavalin A, all decreased the adhesion of *Pseudomonas fragi* to stainless steel. However, Allison and Sutherland (1987) found that adhesion and the presence of polysaccharide were independent, with both a non-polysaccharide-producing mutant as well as the polysaccharide-producing wild type adhering equally well to glass. Similar results were produced when comparing the adhesion of lactococci that differ in the ability to produce polysaccharide (S. H. Flint *et al.*,

unpublished data).

That EPS plays little role in the initial adhesion of cells of thermo-resistant streptococci was supported by these trials which quantified adhesion following treatment of the cells with chemicals that disrupt cell surface polysaccharides. Treatments used were lysozyme, which dissolves bacterial cell wall mucopolysaccharides by hydrolysing the β (1-4) linkages between N-acetyl-amino-2-deoxy-D-glucose residues (Windholz 1983), sodium metaperiodate, which oxidises vicinal hydroxyl groups of component monosaccharides (Gopal and Reilly, 1995), and TCA, which extracts peptidoglycan-associated cell wall polymers (Heckels and Virji, 1988). Measurement of extractable EPS before and after treatment, indicated that these treatments disrupted the polysaccharide as evidenced by an increase in the amount of polysaccahride removed from the cells following the breaking of polymer bonds after chemical treatment. However, none of the three treatments resulted in a difference in the adhesion of thermo-resistant streptococci isolates to stainless steel, even after the disrupted polysaccharides were removed by sonication.

Interestingly, following treatment with polysaccharide-degrading chemicals, there was a range in the ability of different strains of treated cells to adhere with specific treatments resulting in both increases and decreases in the number of specific strains adhering. Specific treatments resulted in both increases and decreases in the number of cells of specific strains adhering. This was not consistent for particular strains for all chemical treatments and could not be related to strains from a particular source. Both *S. thermophilus* and *S. waiu* strains were affected. This heterogeneity in the response of particular strains makes the screening of several species by more than one method important in producing meaningful results.

In biological systems, proteins on the bacterial cell surface mediate adherent interactions with host tissues. For example, site-specific colonisation by oral

streptococci results from an interplay between the host receptor and bacterial adhesion protein expression (Jenkinson, 1994).The importance of proteins in the adhesion of cells to inorganic substrates is less well documented. However, a recent publication on the adhesion of *Azospirillum brasilense* to glass and polystyrene surfaces indicated a correlation between the protein concentration at the cell surface and adhesion density (Dufrêne *et al.,* 1996).

In the present investigation, the effect of protein degrading treatments on the adhesion of thermo-resistant streptococci to stainless steel was determined. The removal of cell surface proteins from the thermo-resistant streptococci using SDS or trypsin led to an observed reduction in the numbers of cells adhering. This suggested that cell surface proteins are important in the initial adhesion phase. To check that SDS or trypsin treatment was not affecting adhesion due to a reduction in cell numbers, counts were carried out on suspensions of treated and untreated cells using a microscopic counting chamber. Counts for treated and untreated cells were similar. The reduction in adhesion was similar for both treatments for each of the 11 isolates tested. These results provide evidence for the involvement of proteins in the initial adhesion of thermo-resistant streptococci. Treatments to remove cell surface proteins (and treatments used to disrupt polysaccharides) kill the bacterial cells; however, a difference in adhesion due solely to loss of viability was discounted as it had been previously established that inactivated cells attached in similar numbers to living cells. The interaction of the cell surface proteins with the substrate may involve electrostatic interactions or affect the hydrophobicity of the cells. The hydrophobicity of *S. thermophilus* cells decreased following treatment with trypsin - a treatment that was shown to reduce adhesion. However, the overall magnitude of the negative charge or hydrophobicity could not be related to the numbers of bacteria adhering. Paul and Jeffrey (1985) found that treatment with proteolytic enzymes decreased the hydrophobicity of *Vibrio proteolytica* as determined by the adhesion to polystyrene and the MATH test. Similarly, in the present trial, treatment with trypsin decreased

the hydrophobicity of *S. thermophilus*. This was probably due to the removal of hydrophobic protein groups from the surface of the cells. However, with the exception of specific forms of bacteria, *e.g. Bacillus* spores (Wiencek *et al.,* 1991), there is no clear evidence in the literature that hydrophobicity is a strong predictor of adhesion to solid surfaces. Hydrophobicity may play a more important role in the advanced stages of colonisation of surfaces as indicated by the predominance of hydrophobic isolates from dairy manufacturing plants (Chapter 4). This may have some role in preventing aqueous solutions from removing biofilms.

To visualise any change in the cell surface following treatment with trypsin, cells were examined by TEM. No bacterial structures such as fibril tufts, which have been observed on oral streptococci and have been implicated in adhesion (Weerkamp *et al.*, 1986), were observed on the surface of *S. thermophilus* (H) before or after treatment. The increased thickness of the cell wall or space between the cell wall and cell membrane, observed in trypsin-treated cells, may have been related to the reduced adhesion. However, this may also have been an artefact due to shrinkage of the cytoplasm during trypsin treatment.

That the proteolytic treatments affected protein was confirmed by SDS-PAGE analysis which identified changes in the cellular proteins following treatment with SDS or trypsin. The most obvious change was the loss of a polypeptide of approximately 55 kDa following treatment with SDS. The N-terminal sequence of this protein matched that of β-lactoglobulin, although the molecular weight of the protein isolated from the thermo-resistant streptococci was three times that of β-lactoglobulin. Extracts of proteins from the cell wall of *S. thermophilus* (H) produced two polypeptides on SDS-PAGE. The different molecular weight of these extracted polypeptides may have been a result of the extraction process as it is difficult to extract molecules of exactly the same molecular weight as the original due to fragmentation of the cell wall (Russell, 1988). The identification of one of these

bands, using antisera to the 55 kDa polypeptide, suggests that this protein band associated with the cell wall fraction may be part of the "adhesion protein". Unfortunately, no N-terminal sequence of this cell wall fraction could be obtained as it appeared to be blocked. This blocking could have been a result of the extraction process.

The inhibition of adhesion in the presence of antibodies to the "adhesion protein" is further evidence of the importance of this "adhesion protein" in the adhesion of thermo-resistant streptococci to a stainless steel substrate. The absence of any reduced adhesion following the exposure of the cells to normal rabbit serum confirmed that this effect was specific to the antibodies present in the antiserum. The association of the "adhesion protein" with the cell surface was demonstrated by observing immunolabelled cells with TEM.

Further evidence of a cellular origin for the "adhesion protein" may be obtained in future studies by designing a nucleic acid probe to the N-terminal sequence and attempting to generate a polymerase chain reaction product from *S. thermophilus* DNA. It may then be possible to inactivate the gene encoding the adhesion protein to provide further evidence for the involvement of this protein in adhesion. However, this technique is not always successful because of pleiotrophic effects or genetic or metabolic compensation by the cell (Jenkinson, 1995).

The possibility of the organisms having acquired the "adhesion protein" from the environment was investigated. The fact that the molecular weight of the "adhesion protein" was three times that of bovine β-lactoglobulin might be explained by the formation of a trimer of β-lactoglobulin, which could occur during the heat treatment of milk and might be carried with the casein peptone fraction of the M17 medium used in the cultivation of these cells. However, this could not be demonstrated in SDS-PAGE of the M17 medium. Any β-lactoglobulin in the M17 medium is likely

to be present in very low concentrations as the only protein of milk origin in this medium is in the polypeptone which contains a casein-based peptone, manufactured from purified casein (Terzaghi and Sandine, 1975). It is possible that traces of contaminating protein were present in concentrations too low to be detected by SDS-PAGE of the M17 medium but were able to be concentrated on the cell surface of thermo-resistant streptococci growing in this medium.

The possibility of the "adhesion protein" being acquired by the cell from the environment (*e.g.* growth medium) was further examined by passaging the cells in synthetic or modified media assured to be free of this protein. The 10-fold reduction in the number of cells, grown in synthetic medium, trypsin digests, or ultrafiltrate variations of the M17 medium, attaching to stainless steel suggests that this was a possibility. However, the 55 kDa band was still present in cells grown in the synthetic media. The synthetic or modified media vary in many respects from the original M17 medium. Even the trypsin digest or ultrafiltrate of M17 medium could vary through the digestion of many different proteins or the removal of many complexes from the medium other than the "adhesion protein".

The "adhesion protein" appears to be similar to β-lactoglobulin - a protein that appears to have an affinity for surfaces. For example, β-lactoglobulin is believed to be involved in fouling in dairy processing plants (Jeurnink *et al.*, 1996). This affinity for surfaces is supported by the observed inhibition of adhesion of *S. thermophilus* (H) to stainless steel that had been exposed to β-lactoglobulin. Here it appears that this protein blocked adhesion sites on the surface, essentially competing with a similar protein associated with the cell surface. Similar observations have been made by Bourassa *et al.* (1996) who found that unidentified whey proteins reduced the adhesion of *Lactococcus lactis diacetylactis.* This disagrees with the hypothesis that substrates are often coated or conditioned with protein which assists in the adhesion of microbial cells (Kirtley and McGuire 1989; Marshall, 1996). Conditioning of

surfaces for the adhesion of bacteria may depend on the type of protein and the bacteria.

The association of adhesion of thermo-resistant streptococci with proteins, having an N-terminal amino acid sequence matching that of β-lactoglobulin is analogous to the binding of *Escherichia coli* heat-labile enterotoxin to milk components (Shida *et al.*, 1994). A 20 kDa protein with an N-terminal sequence matching that of β-lactoglobulin, originating from milk and believed to be formed during the heating of milk, is involved in the adhesion of this toxin to milk components (Shida *et al.*, 1994).

5.5 CONCLUSIONS

This is the first report suggesting the involvement of proteins associated with the cell surface in the adhesion of thermo-resistant streptococci to stainless steel. Treatment of the cells with either SDS or trypsin reduced adhesion. A 55 kDa protein with an N-terminal sequence matching that of β-lactoglobulin was identified as being involved in adhesion. Evidence that this polypeptide is an adhesion protein was obtained from the blocking of adhesion with specific antibodies, by identifying the polypeptide on the surface of the thermo-resistant streptococci by immunolabelling, and by blocking adhesion with β-lactoglobulin, the protein matching the N-terminal of the adhesion polypeptide. The origin of this adhesion protein is probably the microbial cell.

As surface proteins appear to have an influence on the adhesion of these bacteria, proteolytic chemical treatments may be critical in the successful removal of biofilms. It has already been suggested that the initial adhesion bonding the organism to the surface should be the focus for studies on detachment (Busscher *et al.*, 1995b).

Chapter 6

REMOVAL AND INACTIVATION OF THERMO-RESISTANT STREPTOCOCCI FROM STAINLESS STEEL

6.1 INTRODUCTION

The control of biofilms involves frequent cleaning to remove and inactivate the bacteria in the biofilm. Routine cleaning procedures *e.g.* clean-in-place (CIP), in food manufacturing plants generally involve rinsing and chemical cleaning to remove soil, followed by the use of sanitisers or disinfectants to inactivate bacteria that have not been removed by the cleaning step (Romney, 1990). Factors that affect the efficiency of chemical disinfectants are interfering substances (primarily organic matter), pH, concentration, temperature and contact time. Most chemical disinfectants require a minimum contact time of 5 min and the relationship between the death of microorganisms and the disinfectant concentration follows a sigmoid curve (Holah, 1995a). The types of chemical disinfectants suitable for the food industry have been summarised (Holah, 1995b).

Microorganisms in a biofilm often show a greater resistance to antimicrobial substances than those in a planktonic form (Nichols, 1989; Brown and Gilbert, 1993; Te Giffel *et al.*,1997). The mechanism by which adhered cells become resistant to chemical sanitisers is unknown. Possible mechanisms include the following.

(1) Quorum sensing, where the proximity of bacteria to one another generates a mutually protective effect.

(2) Boundary layers on the substrate that restrict molecular movement, thereby restricting the effectiveness of chemical diffusion.

(3) Stress shock proteins that rapidly act to prevent damage to the cell.

(4) Reduced water activity due to one surface of the bacteria being attached to the substrate.

(5) Quenching of the active chemical material due to the organic material (*e.g.* milk protein) associated with the biofilm.

Any of these factors may affect the physiological state of the cell and there is evidence that this is important in the resistance of microorganisms in a biofilm to disinfectants (Carpentier and Cerf, 1993; Yu and McFeters, 1994). Due to the difficulty in inactivating the cells, control may best be achieved by focusing on removal of the bacteria rather than inactivation.

Chemical cleaners that are primarily designed to remove soil have also been reported to remove the majority of microorganisms (Holah, 1995a). However, the choice of cleaning agent is particularly important as cleaning chemicals behave differently. For example, both alkalis and acids will break down proteins but do so via different mechanisms. Alkali cleaners act on hydroxyl ions of proteins and the addition of chlorine aids the removal of proteinaceous deposits even though the bactericidal activity of chlorine is optimum at pH 6.5 (Holah, 1995a). Acids solubilise proteinaceous deposits through hydrolysis (Holah, 1995a).

A general purpose food detergent typically contains the following: a strong alkali to saponify fats, weaker alkali builders or bulking agents, surfactants to improve wetting, dispersion and rinsability, and sequestering agents to control hard water ions (Holah, 1995a). Organic surfactants are composed of a long non-polar chain and a polar head and this enables the dispersion of organic molecules in an aqueous environment. Surfactants are classified as anionic, cationic or non-ionic, depending on their ionic charge in solution. Anionic and non-ionic surfactants are more commonly used than cationic surfactants. Amphipolar molecules aid cleaning by

reducing the surface tension of water and emulsifying fats (Holah, 1995a). Sequestering agents (chelating agents) are used to prevent the precipitation of mineral ions by forming soluble complexes with these ions. The primary use of these agents is in the control of hard water ions, and they are added to surfactants to aid dispersion and rinsability (Holah, 1995a).

Physical as well as chemical factors may influence the effectiveness of cleaning. Wirtanen *et al.* (1993) showed that surface roughness of the stainless steel is the most important factor in removal of biofilms. The importance of turbulent flow (Reynolds number > 3500) in cleaning stainless steel pipework is well established, with flow rates of 1.5 m s^{-1} recommended for most industrial systems (Romney, 1990). However, there is evidence that flow rates play only a small role in improved cleaning in some systems. For example, in membrane cleaning systems, Reynolds numbers of 1500-6000 have been shown to have little effect on cleaning, indicated by only a small increase in flux recovery (Bird and Bartlett, 1995).

The overall cleaning plan will also influence the efficiency of keeping a manufacturing plant clean. For example, it has been demonstrated over sequential manufacturing runs that using routine cleaning (caustic, acid and sanitiser washes) results in accumulation of soil and microorganisms (Dunsmore *et al.*, 1981; Halah, 1995b). However, cleaning efficiency can be enhanced by varying the standard parameters (*e.g.* temperature and the chemicals used and time of exposure), depending on the nature and extent of soiling within a manufacturing plant (Holah, 1995b).

The choice of test bacteria affects the results when comparing different cleaning treatments (Wirtanen *et al.,* 1993). In addition, microorganisms either can be incorporated into the soil or can attach directly to surfaces and form layers of biofilms and may vary in their susceptibility to cleaning treatments within different

environments (Wirtanen *et al.*, 1993).

In this study, on the adhesion of thermo-resistant streptococci to stainless steel have demonstrated that these bacteria readily attach to the substrate in the absence of milk proteins. The inhibition of attachment in the presence of milk proteins and the predominance of thermo-resistant streptococci in the cooling section of a pasteuriser - outside the region where fouling with milk protein occurs (Bouman *et al.*, 1982) - suggest that control measures must focus on the removal of the bacteria rather than rely on removal of soil to remove the bacteria. This thesis has also presented data demonstrating the importance of cell-associated proteins in adhesion. Therefore, if thermo-resistant streptococci resist current cleaning regimes and survive exposure to sanitisers, more effective proteolytic cleaning treatments need to be studied in order to develop or devise methods to remove these bacteria from the substrate.

This chapter reports on the sensitivity to sanitisers of thermo-resistant streptococci in planktonic and adhered forms and the removal of thermo-resistant streptococci from stainless steel substrates using different chemical treatments.

6.2 PROCEDURES

Streptococcus thermophilus (H) and (48) (Section 3.1) were used in experiments to determine the effect of heat and chemicals on the viability of cells in planktonic, adhered and biofilm forms and the effect of chemicals in removal of cells from stainless steel substrates.

To determine the sensitivity of thermo-resistant streptococci to heat, samples of planktonic or adhered cells were heat treated in plastic "Whirl Pak" bags (Biolab Scientific, Palmerston North, New Zealand) in a water bath (Bremer and Osborne, 1997) (Section 3.7.1). Heat treatment in test tubes (Section 3.7.2) was compared with

the plastic bag method. Cell viability was determined using the Malthus microbiological growth analyser (Section 3.4.2). Thermal death curves were prepared from the regression of the viable cell numbers against time for each heat treatment temperature (SigmaPlot, Jandel Corporation, San Rafael, California, USA) The D-values (time in minutes required for a 10-fold reduction in the numbers of microorganisms at a given temperature) were determined from the negative reciprocal of the slope of each thermal death curve. D-values at 60 and 64°C for strains (H) and (48) from thermal death data obtained using the test tube method were compared with the D-values obtained using the plastic bag method.

The plastic bag method was selected from preliminary data as the most accurate method for this study. Thermal death curves at 56, 58, 60, 62 and 64°C were prepared for planktonic cells of *S. thermophilus* (H) in water. Thermal death curves at 58, 60, 62, 64 and 66°C were prepared for adhered cells of *S. thermophilus* (H) in water (Section 3.7.1). Thermal death data at 60, 62 and 64°C for cells adhered in water were compared with data for cells adhered in the presence of sterile skim milk, and then heat treated following thorough rinsing with water. Biofilms of *S. thermophilus* (H) that were 12 and 24 h old and prepared in a laboratory reactor (Section 3.3.2), were heat treated at 60°C and the D-values were compared with those from planktonic and recently adhered cells.

A similar series of heat treatment trials was carried out using *S. thermophilus* (48). Thermal death curves at 60, 62, 64 and 66°C were prepared for planktonic cells and at 62, 64 and 66°C for for adhered cells.

The Z-values (change in temperature (°C) required to produce a 10-fold change in the D-value) were determined for both planktonic and adhered cells of *S. thermophilus* (H) and (48) from the negative reciprocal of the slope of each graph of $\log_{10}$ D-values *vs* temperature.

The sensitivity to chemical sanitisers of planktonic, recently adhered or biofilm cells of thermo-resistant streptococci was determined by adding cells, or substrate colonised with cells, to known concentrations of chlorine or quaternary ammonium-based sanitisers (Section 3.7.3). The cells or substrates colonised with cells were rinsed but no inactivators were used to inactivate the sanitisers as this is not practice in dairy manufacturing plants. Viability was determined after 30 min exposure using the Malthus microbiological growth analyser (Section 3.4.2).

The effect of standard dairy industry cleaning and sanitising procedures in removing and inactivating naturally occurring biofilms in a dairy manufacturing line was determined by monitoring a dairy manufacturing plant using a specially designed modified Robbins device (MRD) (Section 3.3.1). This was installed in-line immediately following the regeneration section of the pasteuriser and remained there for the duration of the experiment. Temperatures in this region were 37-40°C (ideal for the growth of thermo-resistant streptococci). The pasteuriser processed the same amount of milk each week and was cleaned after 6 h of operation using a CIP system consisting of a water flush, 0.8% NaOH for 45 min, a water rinse, 0.6% HNO_3 for 45 min and a final water rinse at temperatures above 70°C. At weekly intervals, over 15 weeks, four coupons were removed from the MRD before and after cleaning and eight fresh coupons were aseptically installed. The total numbers of living and dead cells within the biofilm associated with the surface were determined by a combination of epifluorescence microscopy (two coupons examined before cleaning and two coupons examined after cleaning) and conductance techniques (two coupons examined before cleaning and two coupons examined after cleaning). Samples were taken weekly over 15 weeks, before and after cleaning.

The effects of different chemicals (trypsin, sodium dodecyl sulphate (SDS), trichloroacetic acid (TCA), sodium metaperiodate ($NaIO_3$), lysozyme, mutanolysin,

NaOH and HNO_3) and control procedures (samples washed with water or treated at 100°C for 15 min) were compared for the removal biofilm cells of thermo-resistant streptococci from stainless steel. Biofilms were generated in the laboratory reactor on 1 cm^2 samples of stainless steel (Section 3.3.2). Biofilms of *S. thermophilus* (H) that were 4 and 18 h old were treated by exposure to chemicals in test tubes under optimum conditions (Section 3.7.4).

A commercial enzyme cleaner "Paradigm" (Ecolab, Hamilton, New Zealand) was tested on 20 h biofilms using a test tube method (Section 3.7.5). The numbers of cells colonising the substrate before and after cleaning were determined using epifluorescence microscopy (Section 3.4.1).

Laboratory scale tests on the commercial cleaner, "Paradigm", were followed with tests in a pilot scale plant (Section 3.7.6) using biofilms prepared on seeded sample disks (Section 3.7.6). The number of cells colonising the surface was determined by epifluorescence microscopy (Section 3.4.1) and conductance microbiology (Section 3.4.2).

6.3 RESULTS

6.3.1 Sensitivity to heat

To determine the sensitivity of thermo-resistant streptococci to heat, two different test methods using either plastic bags or test tubes were compared. The D-values for planktonic cells using both methods were similar. However, the D-values for adhered cells using the tube method were two-fold greater than those obtained using the plastic bag method (Table 6.1). The bag method was selected for all studies to avoid possible problems in heat transfer to immobilised cells, believed to explain the difference in results for adhered cells using the tube method.

The thermal death curves for *S. thermophilus* (H) as planktonic or adhered cells in water produced similar D-values (Figures 6.1 and 6.2) (Table 6.2). The Z-values, determined from the regression of the $\log_{10}$D-values against temperature (Figure 6.3), were greater for the adhered cells (10.0°C for planktonic cells and 12.0°C for adhered cells) (Table 6.5). The calculated D-values from Figure 6.3 were compared with the observed results in Table 6.3. The equations derived from Figure 6.3 are given below:

$$\textit{Planktonic cells} \quad Log\ D = -0.101(t) + 6.528 \quad r^2 = 0.92$$

$$\textit{Adhered cells} \quad Log\ D = -0.0825(t) + 5.375 \quad r^2 = 0.93$$

Where D = D-value (time in minutes required for a 10-fold reduction in the numbers of microorganisms)

t = temperature (°C)

r^2 = regression value

Table 6.1

A comparison between two different heat treatment methods - using *S. thermophilus* (H)

Cell type	Temperature (°C)	D-values (min)	
		Tube method	Bag method
Planktonic	60	2.9	2.0
Planktonic	64	1.2	1.3
Adhered	60	4.6	2.2
Adhered	64	2.7	1.3

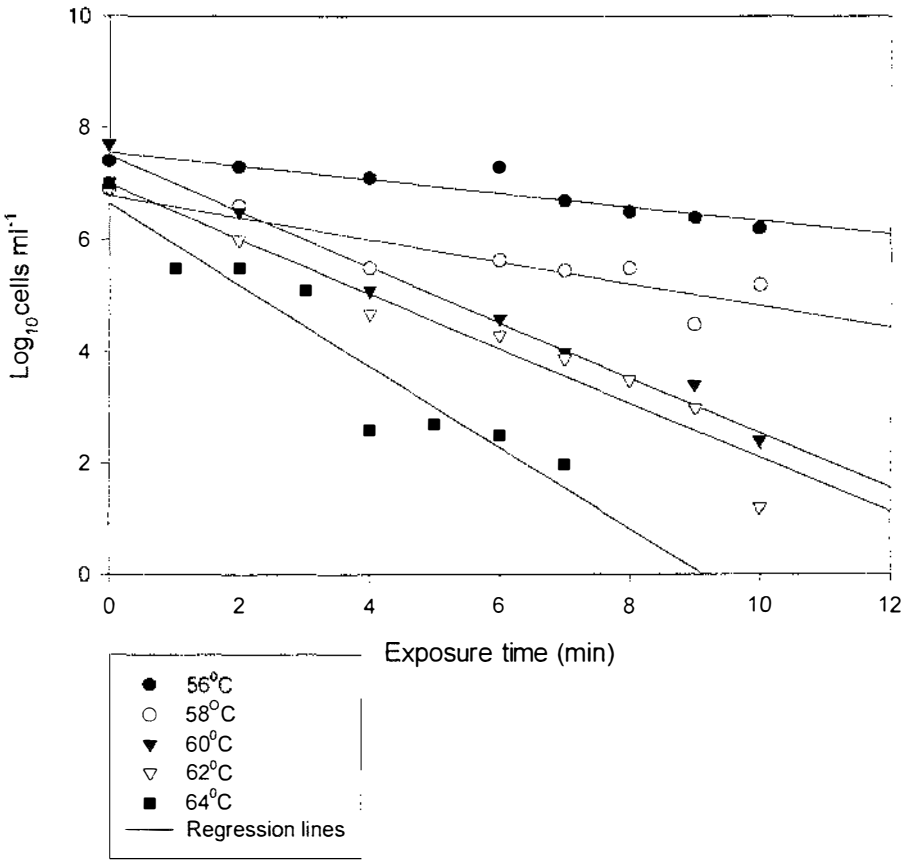


Figure 6.1 Thermal death curves for planktonic cells of *S. thermophilus* (H).

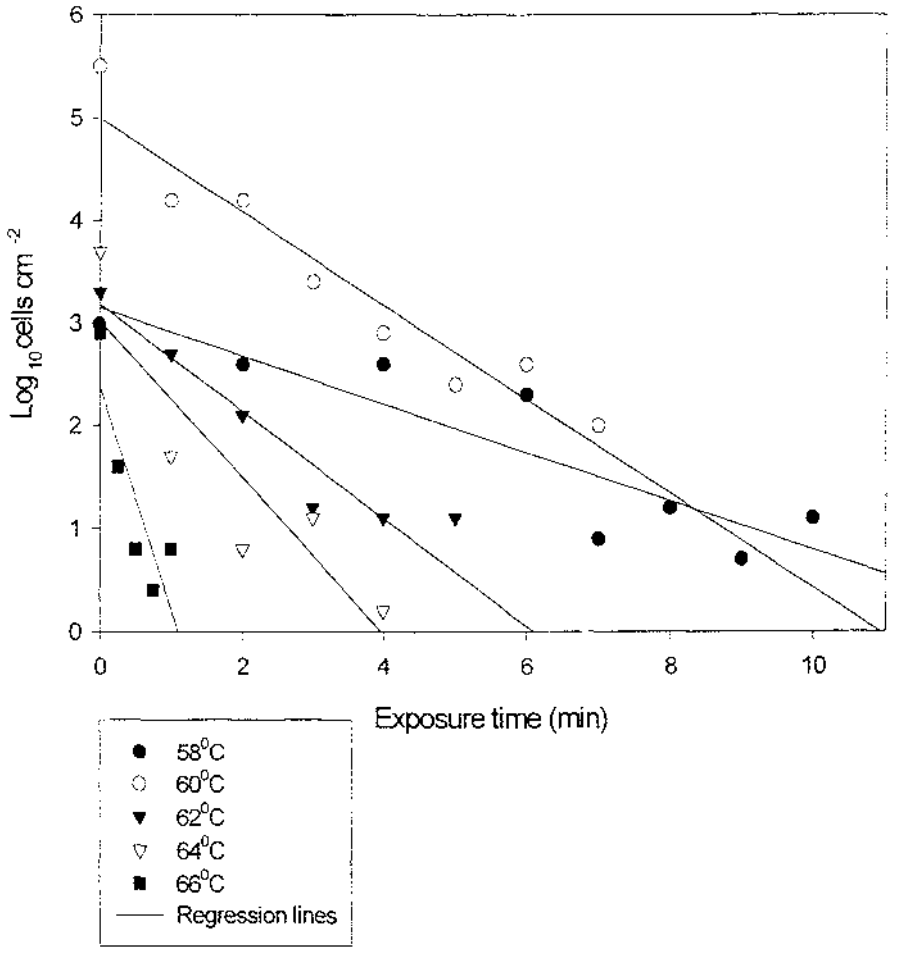


Figure 6.2 Thermal death curves for adhered cells of *S. thermophilus* (H).

Table 6.2

Summary of thermal death curve data for *S. thermophilus* (H) as planktonic and adhered cells

Temp. (°C)	Planktonic cells				Adhered cells			
	Y intercept	Slope	r^2	D-value (min)	Y intercept	Slope	r^2	D-value (min)
56	7.55	-0.12	0.82	8.3	ND	ND	ND	ND
58	6.78	-0.19	0.86	5.3	3.15	-0.23	0.81	4.4
60	7.49	-0.49	0.98	2.0	5.00	-0.46	0.92	2.2
62	6.99	-0.49	0.93	2.0	3.18	-0.52	0.94	1.9
64	6.65	-0.73	0.90	1.3	3.02	-0.76	0.80	1.3
66	ND	ND	ND	ND	2.38	-2.15	0.83	0.5

ND = not done.

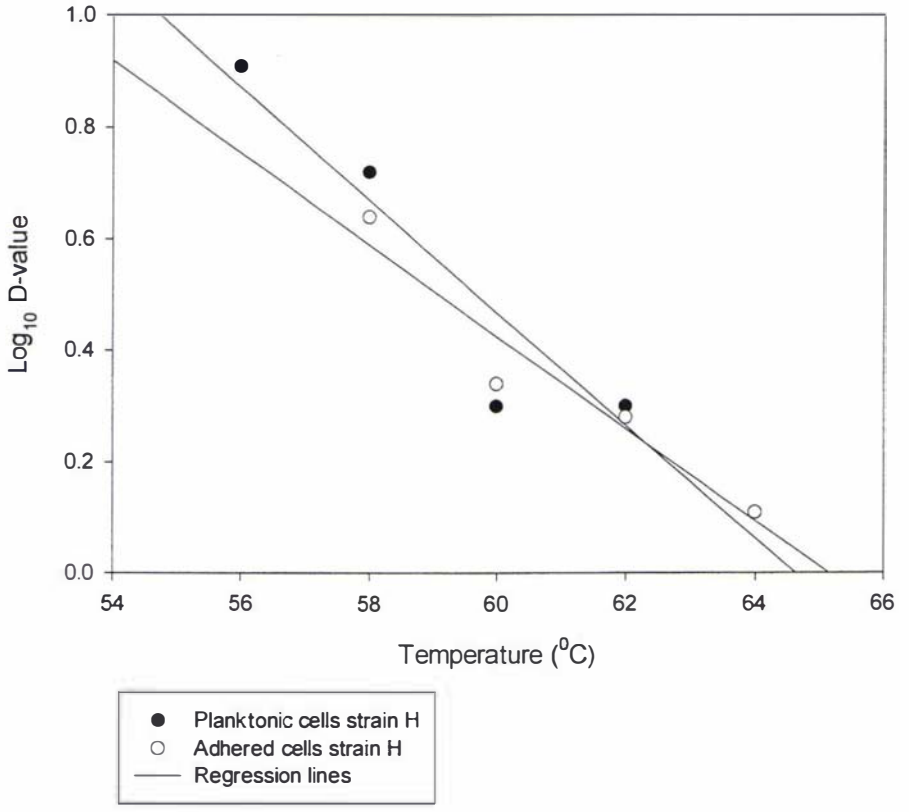


Figure 6.3 Regression of the $\log_{10}$ D-values for planktonic and adhered cells of *S. thermophilus* (H).

Table 6.3

Comparison between the observed and calculated D-values (min) for planktonic and adhered cells of *S. thermophilus* (H)

Temperature (°C)	Planktonic cells D-values (min)		Adhered cells D-values (min)	
	Observed	Calculated	Observed	Calculated
56	8.3	7.5	ND	5.7
58	5.3	4.7	4.4	3.9
60	2.0	2.9	2.2	2.7
62	2.0	1.8	1.9	1.8
64	1.3	1.1	1.3	1.3
66	ND	0.7	0.5	0.9

ND = not done

The D-values of planktonic cells increased in the presence of skim milk compared with cells in the presence of water. Cells that were allowed to adhere in the presence of skim milk but washed in water before heat treatment also showed increased resistance to heat compared with cells allowed to adhere in the presence of water (Figure 6.4) (Table 6.4). For planktonic cells of *S. thermophilus* (H) in skim milk, a seven-fold increase in the D-value at 60°C was recorded compared with the same cells in water (Table 6.4). For cells adhered in the presence of skim milk then washed, a five-fold increase in the D-value at 60°C was recorded compared with planktonic cells in water.

The D-values for biofilms of *S. thermophilus* (H) grown in the presence of skim milk over 12 or 24 h were less than those for cells recently adhered in the presence of skim milk (Table 6.4) (Figure 6.4). The correlation coefficients (r^2) for the thermal death curves produced where skim milk was involved were lower than those for planktonic or adhered cells treated in water.

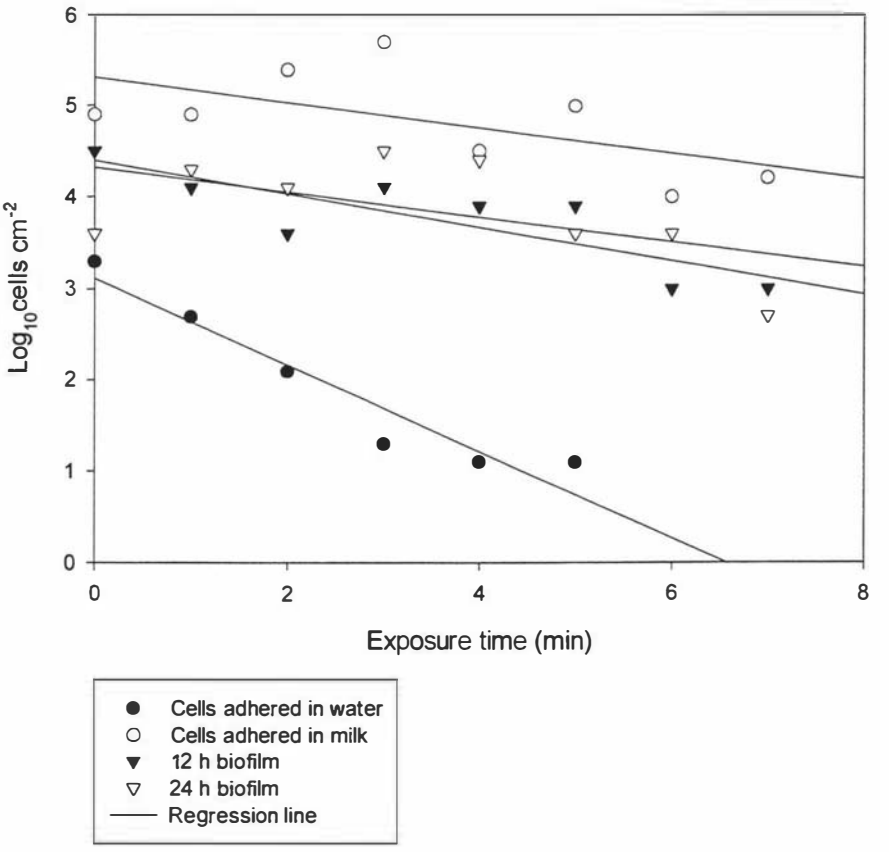


Figure 6.4 Thermal death curves at 60°C for *S. thermophilus* (H) cells adhered to stainless steel in the presence of milk or water and 12 or 24 h biofilms.

Table 6.4

Summary of thermal death curve data at 60°C - the effect of milk and surface growth on the sensitivity of *S. thermophilus* (H) to heat

Description	Y intercept	Slope	r^2	D-value (min)
Planktonic cells in milk	7.34	-0.07	0.43	14.3
Planktonic cells in water	7.49	-0.49	0.98	2.0
Cells adhered in presence of milk	4.6	-0.10	0.50	10
Cells adhered in presence of water	5.0	-0.46	0.92	2.2
12 h biofilm	5.31	-0.14	0.35	7.1
24 h biofilm	4.4	-0.18	0.69	5.5

The thermal death curves were compared for *S. thermophilus* (H) and *S. thermophilus* (48). The results for both strains showed similar trends with slightly lower D-values produced for adhered cells compared with planktonic cells. This indicates that adhered cells may be more sensitive to heat than planktonic cells (Table 6.5). However, the D-values for the planktonic cells of *S. thermophilus* (48) were greater than those obtained for *S. thermophilus* (H), indicating that strain 48 may be more resistant to heat than strain H. The Z-values for adhered cells of *S. thermophilus* (48) were approximately two-fold greater than for the planktonic cells, indicating that these cells are more resistant to changes in temperature than changes in the time of exposure to a particular temperature (Figure 6.7) (Table 6.5). A similar trend was observed for the adhered cells of *S. thermophilus* (H). This suggests that changes in temperature to achieve a 10-fold change in the D-value are greater for adhered cells than planktonic cells.

In summary, adhered and planktonic cells varied in their sensitivity to heat, with adhered cells showing marginally greater sensitivity than planktonic cells to the time of exposure and planktonic cells showing greater sensitivity than adhered cells to changes in the temperature.

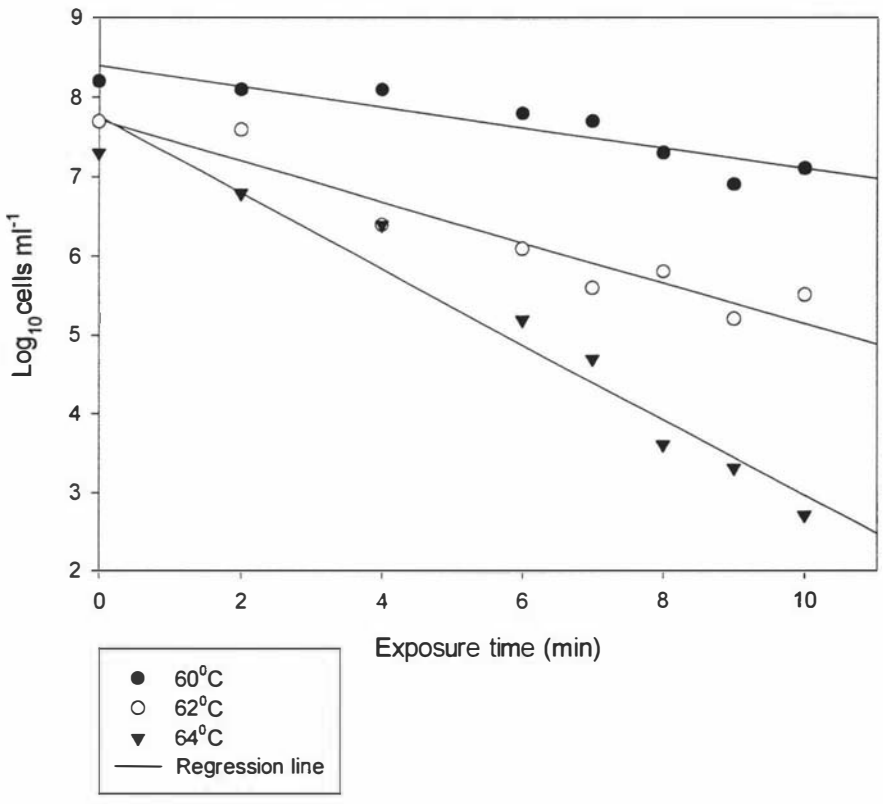


Figure 6.5 Thermal death curves for planktonic cells of *S. thermophilus* (48).

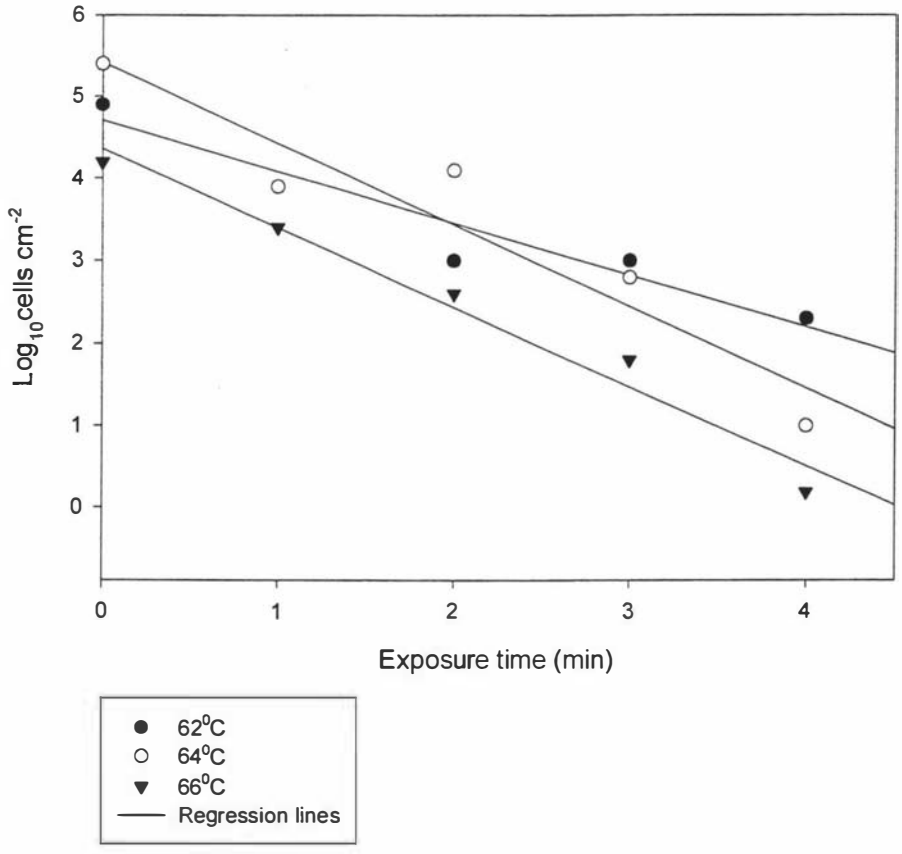


Figure 6.6 Thermal death curves for adhered cells of *S. thermophilus* (48).

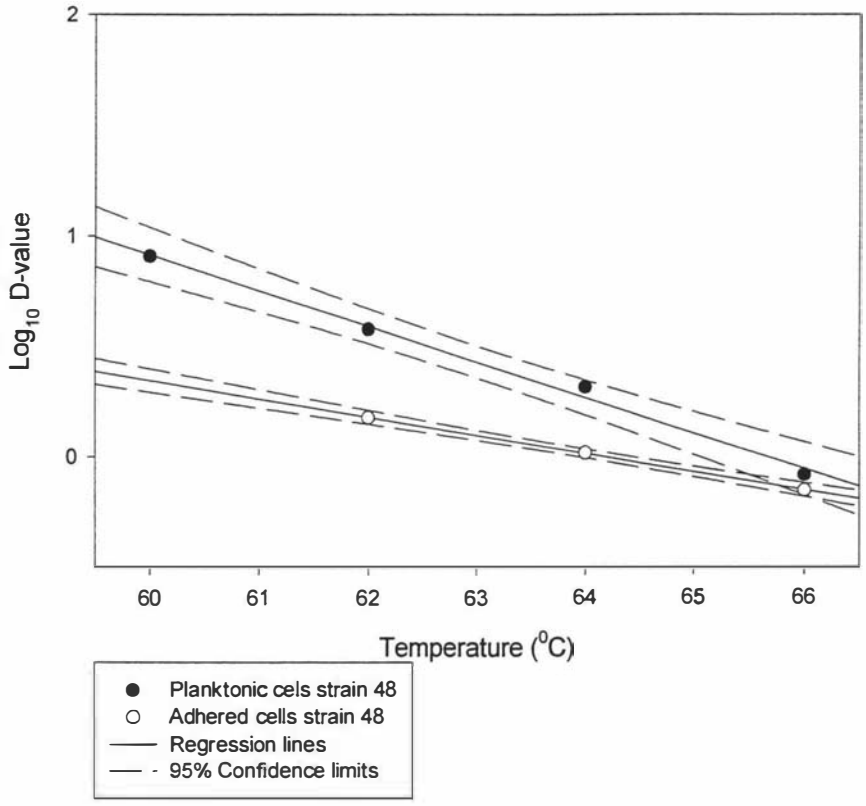


Figure 6.7 Regression of $\log_{10}$ D-values for *S. thermophilus* (48). (Note: only three values for adhered cells therefore 95% confidence limits included.)

Table 6.5

Summary of the D- and Z-values for *S. thermophilus* (H) and (48)

Strain and form	D-values (min)						Z-values (°C)
	56	58	60	62	64	66	
H planktonic	8.3	5.3	2.0	2.0	1.3	ND	10.0
H adhered	ND	4.4	2.2	1.9	1.3	0.47	12.0
48 planktonic	ND	ND	8.3	3.8	2.1	0.84	6.2
48 adhered	ND	ND	ND	1.52	1.06	0.71	12.1

ND = not done.

6.3.2 Sensitivity to sanitisers

The sensitivity of *S. thermophilus* to sodium hypochlorite and cetyltrimethylammonium bromide (CTAB) varied with the form (planktonic, adhered or biofilm) and strain of the cells (Figures 6.8, 6.9, 6.10. 6,11, 6.12 and 6.13).

The sensitivity of planktonic cells of both strains to either sanitiser was similar (Figures 6.8 and 6.9).

The sensitivity of adhered cells to sanitisers varied with the strain. The sensitivity of recently adhered cells of both strains was similar to the sensitivity of planktonic cells when exposed to sodium hypochlorite. Although some recently adhered cells of strain 48 survived 400 ppm sodium hypochlorite, a reduction of over 1000-fold reduction occurred from 20 ppm. Recently adhered cells of strain H were very resistant to CTAB surviving exposure up to 1000 ppm. However, the recently adhered cells of strain H showed a similar sensitivity to CTAB as the planktonic cells (Figures 6.10 and 6.11)

The biofilm cells of both strains showed increased resistance to both sanitisers compared with the planktonic cells. Biofilm cells of strain H were more resistant to CTAB than sodium hypochlorite, surviving 1000 ppm and 400 ppm respectively. Biofilm cells of strain 48 were resistant to 1000 ppm of both sanitisers (Figures 6.12 and 6.13).

6.3.3 Industry monitoring - effect of routine cleaning on biofilms

Routine cleaning (caustic and acid) in a dairy manufacturing plant did not eliminate cells that had colonised the stainless steel surface. The results are detailed in Chapter 4. The total numbers of cells (living and dead) on the surface of sample disks removed over 15 one-week sampling periods were similar (3-4 $\log_{10}$ cells cm^{-2}) before and after cleaning (Figure 4.1). The number of viable cells detected at the same sampling times were 10-fold to 100-fold less than the total number of cells (Figures 4.2 and 4.3) indicating some loss of viability during manufacture, cleaning or sampling.

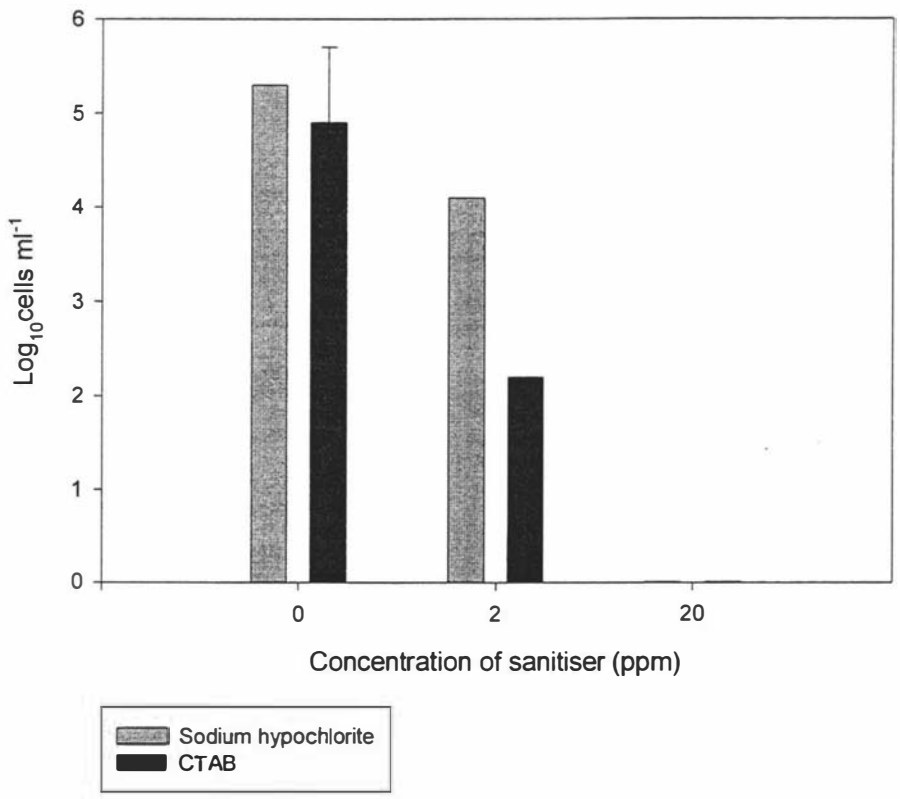


Figure 6.8 The number* of planktonic cells of *S. thermophilus* (H) detected after exposure of the cells to sodium hypochlorite or CTAB at 22°C for 30 min.

* Mean +/- standard deviation. Detection limit using the Malthus microbiological growth analyser approximately 10 cells ml^{-1}.

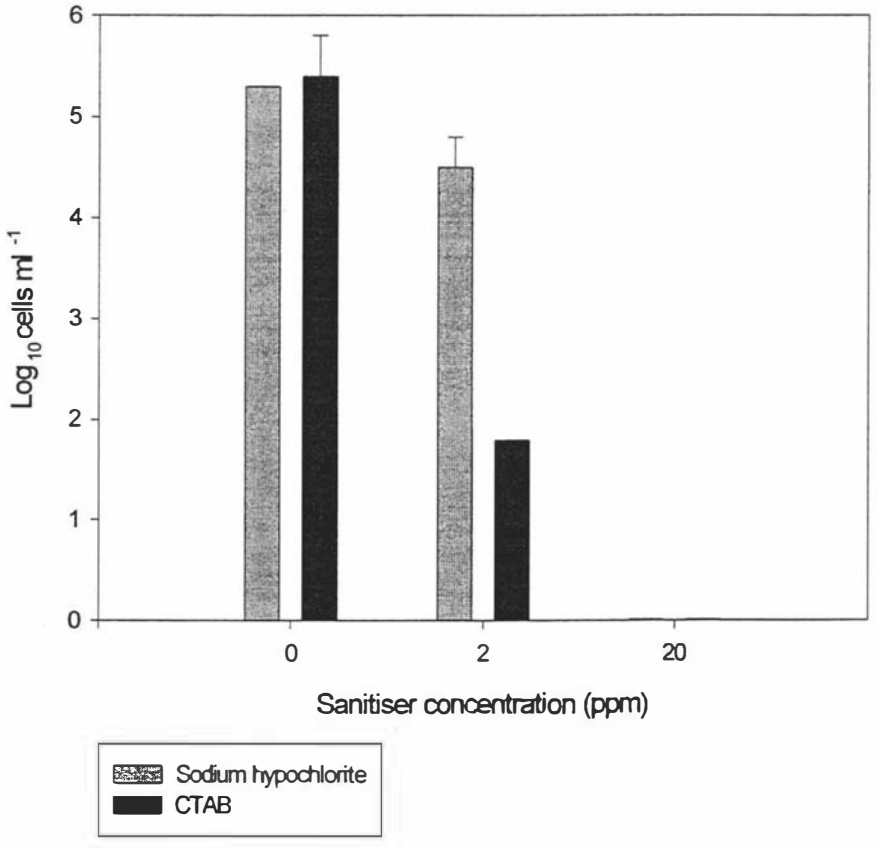


Figure 6.9 The number* of planktonic cells of *S. thermophilus* (48) detected after exposure of the cells to sodium hypochlorite or CTAB at 22°C for 30 min.

* Mean +/- standard deviation. Detection limit using the microbiological growth analyser approximately10 cells ml^{-1}.

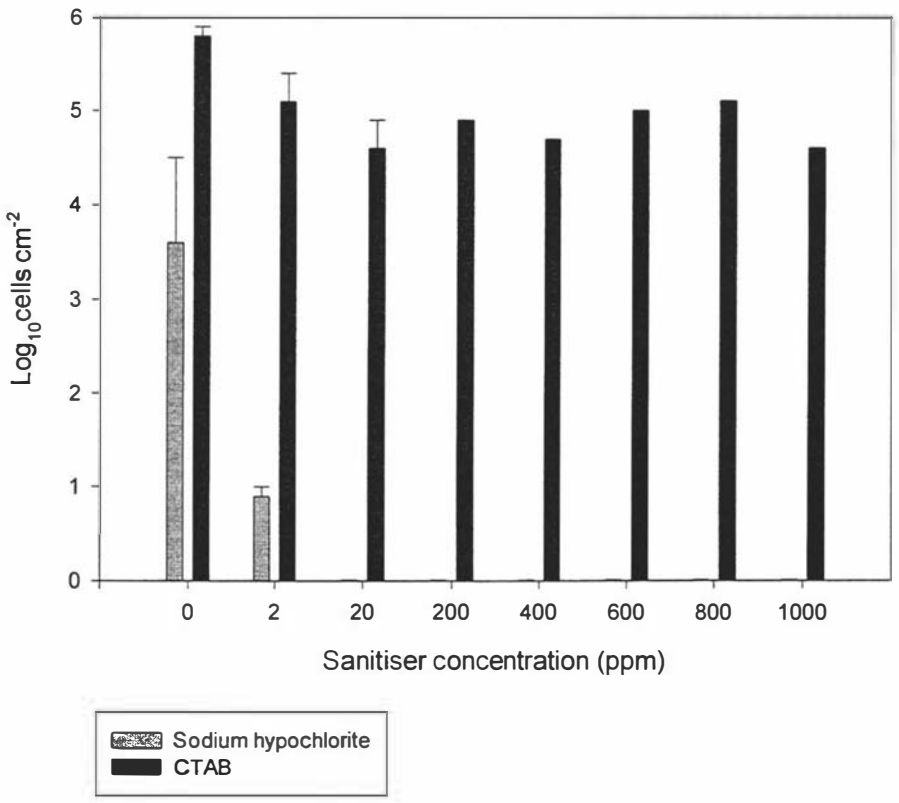


Figure 6.10 The number* of adhered cells of *S. thermophilus* (H) detected on the surface of stainless steel coupons after exposure to sodium hypochlorite or CTAB at 22°C for 30 min.

* Mean +/- standard deviation. Detection limit using the Malthus microbiological growth analyser approximately 1 cell cm^{-2}.

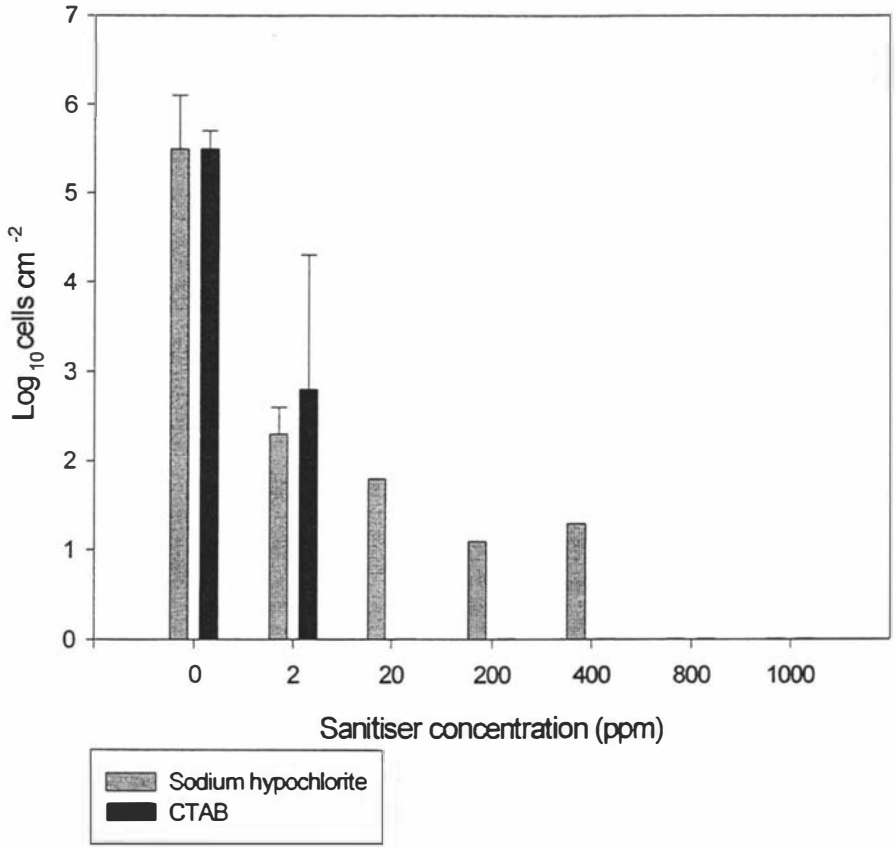


Figure 6.11 The number* of adhered cells of *S. thermophilus* (48) detected on the surface of stainless steel coupons after exposure to sodium hypochlorite or CTAB at 22°C for 30 min.

* Mean +/- standard deviation. Detection limit using the Malthus microbiological growth analyser approximately 1 cell cm^{-2}.

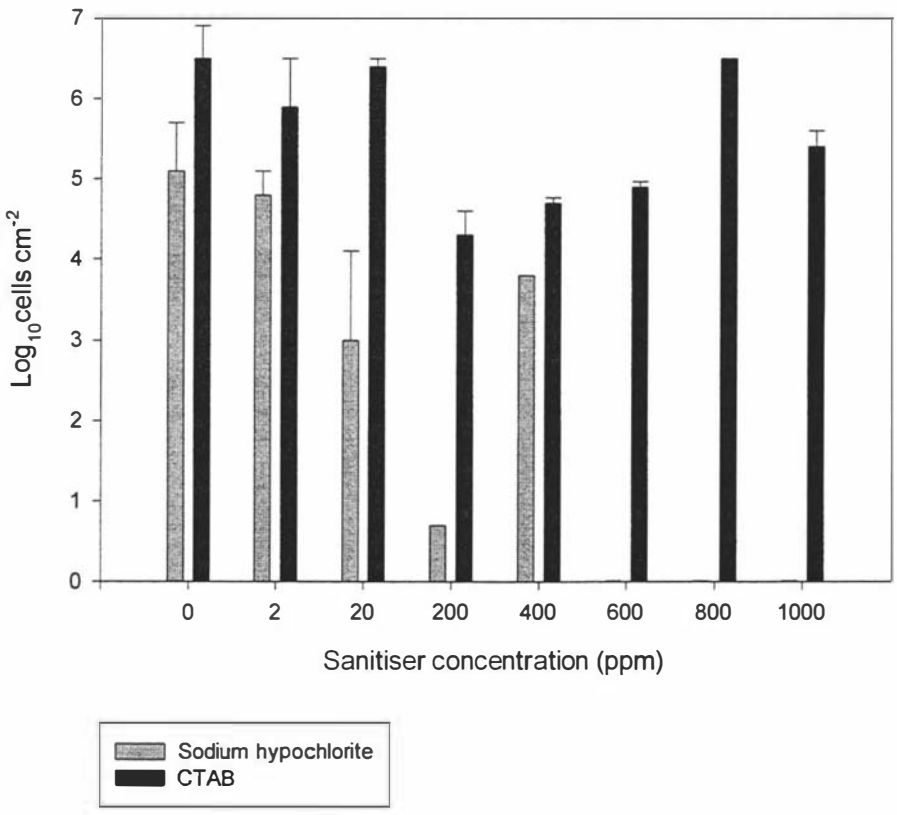


Figure 6.12 The number* of 18 h biofilm cells of *S. thermophilus* (H) detected on the surface of stainless steel coupons after exposure to sodium hypochlorite or CTAB at 22°C for 30 min.

* Mean +/- standard deviation. Detection limit using the Malthus microbiological growth analyser approximately 1 cell cm^{-2}.

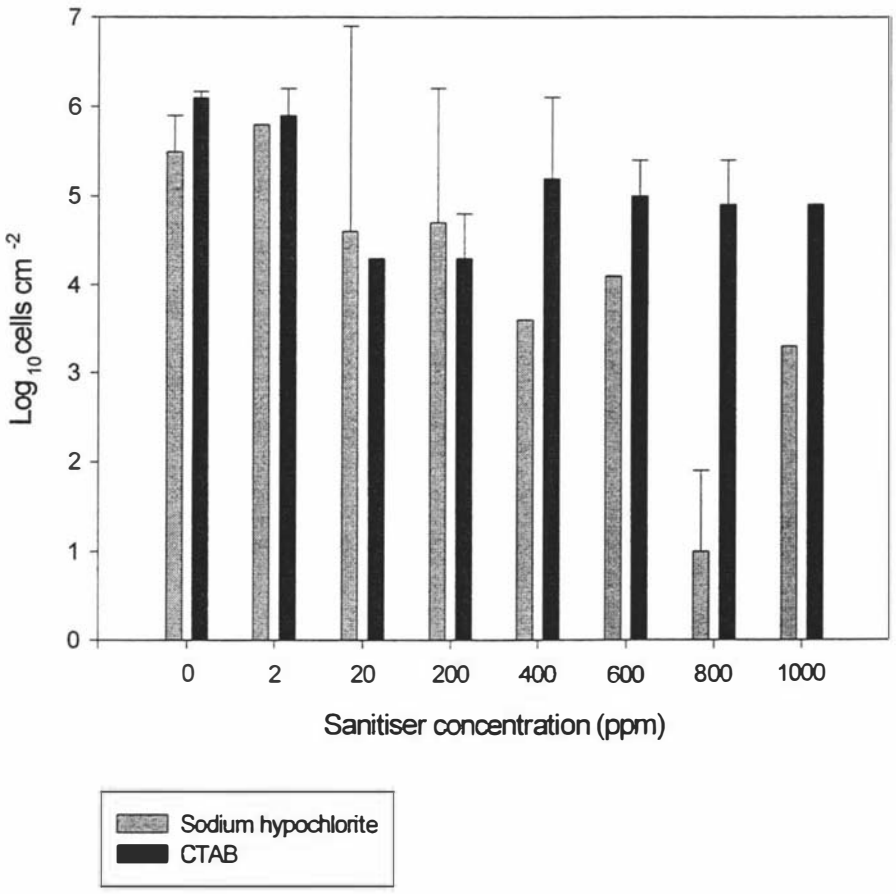


Figure 6.13 The number* of 18 h biofilm cells of *S. thermophilus* (48) detected on the surface of stainless steel coupons after exposure to sodium hypochlorite or CTAB at 22°C for 30 min.

* Mean +/- standard deviation. Detection limit using the Microbiological growth analyser approximately 1 cell cm^{-2}.

6.3.4 Laboratory trials using different chemical treatments to remove biofilms

The effect of 10 different treatments in removing thermo-resistant streptococci colonising the surface of stainless steel was determined, using epifluorescence microscopy. Trypsin treatment was the most effective treatment, reducing the numbers of recently adhered cells or 4 or 18 h biofilm cells by approximately 100-fold (Figure 6.14). Other protein-degrading treatments (*e.g.* SDS, HNO_3 and NaOH) reduced the number of adherent cells by varying amounts, depending on the age of the biofilm. Polysaccharide-degrading agents ($NaIO_3$, lysozyme, and mutanolysin) had negligible effect on any of the biofilms.

Cells in biofilms of thermo-resistant streptococci prepared over 20 h in the laboratory reactor were reduced by a minimum of 100-fold following treatment with a commercial cleaner consisting of proteolytic enzymes ("Paradigm") (Figure 6.15).

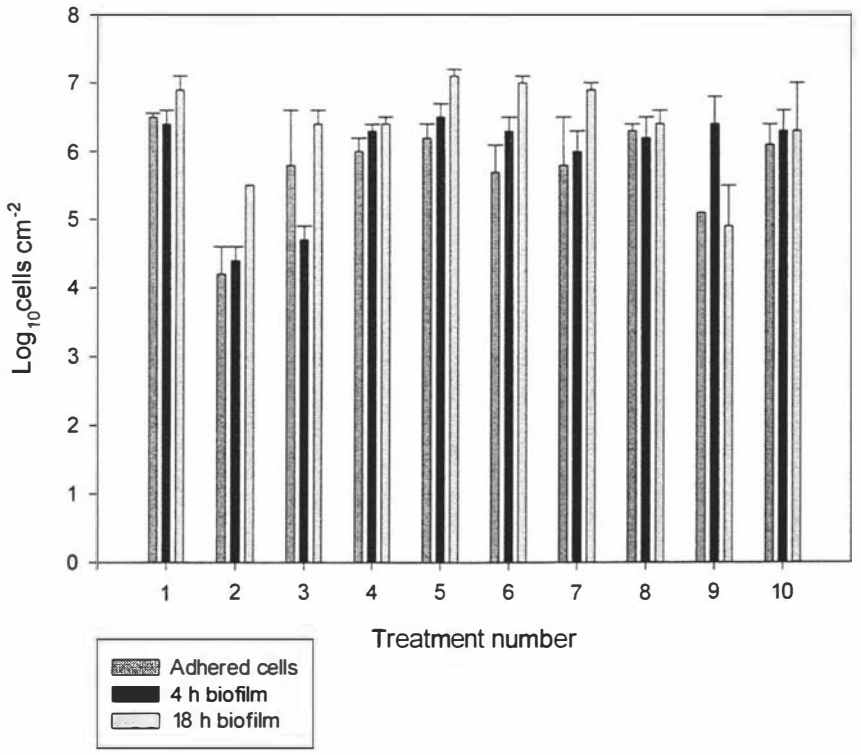
6.3.5 Pilot plant trial to evaluate an enzyme cleaner in removing biofilms

A trial in a pilot-scale pasteuriser was used to evaluate further the effectiveness of a commercial enzyme-based cleaner "Paradigm" in removing biofilms of thermo-resistant streptococci. The total numbers of bacteria present on the plant surface before and after cleaning were estimated using epifluorescence microscopy and varied from 4.6 to 6.0 $\log_{10}$cells cm^{-2} (Figure 6.16). There was a small reduction (< 10-fold) in the total number of bacteria following cleaning with acid and caustic cleaners. The total number of cells reduced by approximately 100-fold following cleaning with "Paradigm".

There was a greater fluctuation in the numbers of viable cells before cleaning (Figure

6.17), determined by conductance microbiology (3.2 to 6.4 $\log_{10}$cells cm^{-2}) compared with the total number of bacteria (4.6 to 6.0 $\log_{10}$cells cm^{-2}) (Figure 6.16). After cleaning with acid and caustic, viable cells were still detected on the sample disks removed from the plant. However, after cleaning with "Paradigm", no viable cells were detected on the sample disks.

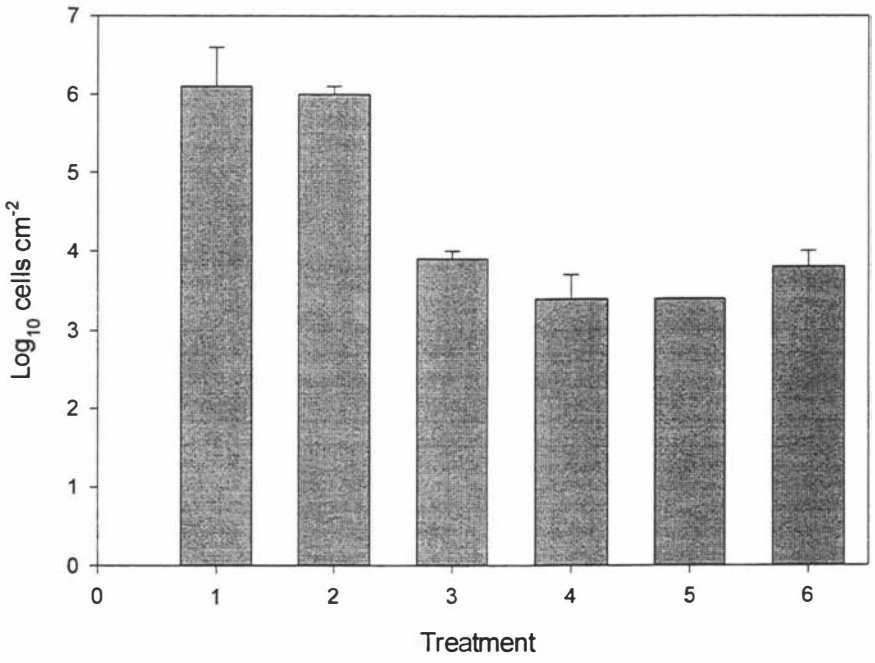
These results supported the results from laboratory trials showing improved cleaning (removal of bacteria from the substrate) using an enzyme-based cleaner. In addition, "Paradigm" was effective in killing all cells on the surface (detection limit approximately 1 cell cm^{-2}).



1 = untreated (washed with water)
2 = 1% trypsin, 37°C 24 h.
3 = 2% SDS, 100°C 15 min.
4 = 10% TCA, 100°C 15 min.
5 = 0.02% $NaIO_3$, 22°C 24 h
6 = 1% lysozyme, 37°C 60 min
7 = 1 un ml^{-1}mutanolysin, 37°C 60 min.
8 = 2% NaOH, 75°C 30 min.
9 = 1.8% HNO_3, 75°C 30 min.
10 = 100°C 15 min

Figure 6.14 The number* of recently adhered and biofilm cells of *S. thermophilus* (H) remaining on the surface of stainless steel coupons after exposure to 10 different treatments.

* Mean +/- standard deviation. Detection limit using epifluorescence microscopy approximately 10^3 cells cm^{-2}.



1 = No treatment.

2 = 1.6% NaOH, 75°C 30 min.

3 = 0.08% Paradigm 2010 and 0.0% 2030, 60°C 30 min.

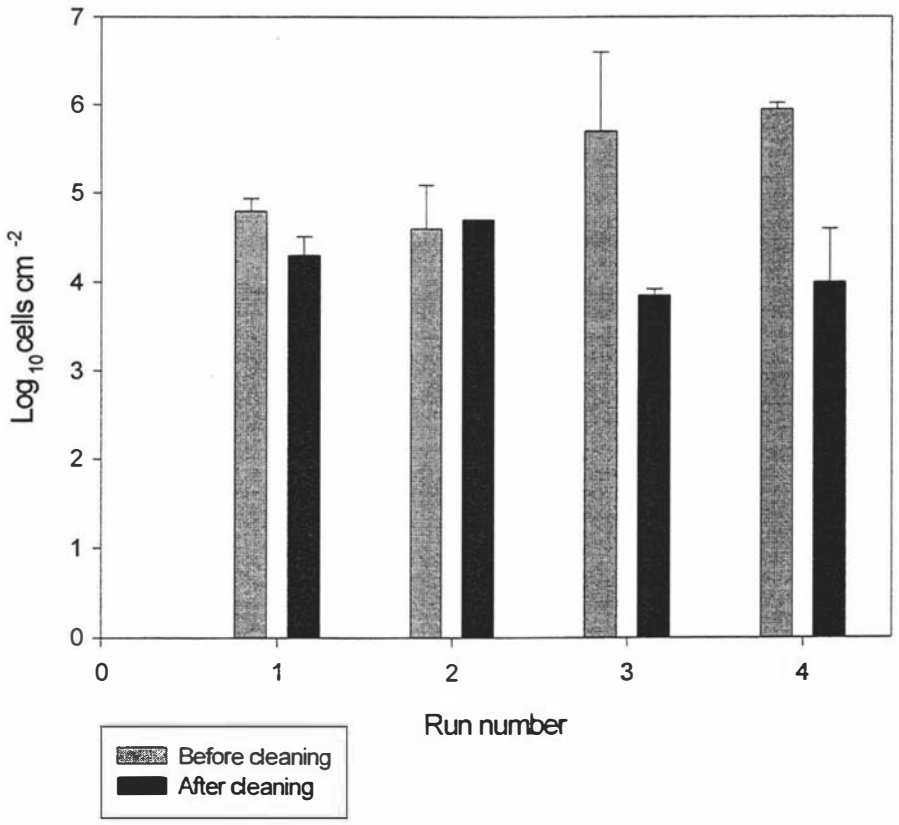
4 = Treatment "2" followed by treatment "3".

5 = 0.08% Paradigm 2010, 60°C 30 min then 0.09% 2030, 60°C 30 min.

6 = 0.08% Paradigm 2010 and 0.09% 2030, 60°C 30 min then 0.8% HNO_3, 75°C 30 min.

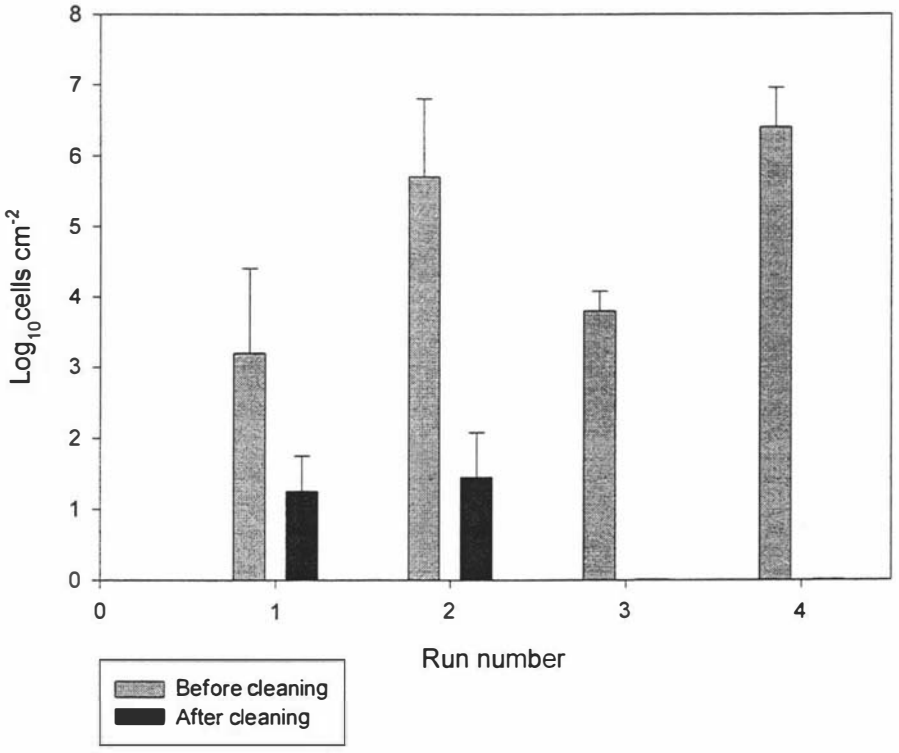
Figure 6.15 The number* of 20 h biofilm cells of *S. thermophilus* (H) remaining on the surface of stainless steel coupons after treatment with "Paradigm", a commercial cleaner containing proteolytic enzymes.

* Mean +/- standard deviation. Detection limit using epifluorescence microscopy approximately 10^3 cells cm^{-2}.



1 = 1.8% NaOH 75°C 30 min; 1.0% HNO_3 75°C 30 min.
2 = 1.8% NaOH 75°C 30 min.
3 = 0.08% Paradigm 60°C 30 min; 1.8% NaOH 75°C 30 min.
4 = 0.08% Paradigm 60°C 30 min; 1.0 % HNO_3 75°C 30 min.

Figure 6.16 The number* of biofilm cells of *S. thermophilus* (H) remaining on the surface of stainless steel from a pilot plant following cleaning. * Mean +/- standard deviation. Detection limit using epifluorescence microscopy approximately 10^3 cells cm^{-2}.



1 = 1.8% NaOH 75°C 30 min; 1.0% HNO_3 75°C 30 min.
2 = 1.8% NaOH 75°C 30 min.
3 = 0.08% Paradigm 60°C 30 min; 1.8% NaOH 75°C 30 min.
4 = 0.08% Paradigm 60°C 30 min; 1.0% HNO_3 75°C 30 min.

Figure 6.17 The number* of viable biofilm cells of *S. thermophilus* (H) remaining on the surface of stainless steel from a pilot plant following cleaning.

* Mean +/- standard deviation. Detection limit using the Malthus microbiological growth analyser approximately one cell per sample disk.

6.4 DISCUSSION

This study investigated the sensitivity of biofilms of thermo-resistant streptococci to bactericides (heat and sanitisers) and the effectiveness of routine cleaning programmes and different cleaning chemicals. Improved control through more effective cleaning methods derived from new knowledge of the adhesion mechanism of thermo-resistant streptococci (Chapter 5) was investigated.

The methodology to evaluate the heat resistance of adhered cells was investigated. A comparison between two different methods to determine heat sensitivity showed considerable variation in the results for adhered cells using the tube and bag methods. Although the lag times to reach temperature (60-64°C) differed (30 s for the bag method; 60-90 s for the tube method), the time of exposure was taken from the time the set temperature was attained. The extra lag time for the tube method; would expose the cells to some form of heat stress for longer than the bag method therefore lower D-values were expected for the tube method. However, for adhered cells, larger D-values were obtained with the tube method than the bag method. This may be explained by the convection currents in the test tubes. For planktonic cells, convection will enable an even exposure to heat as the cells move in the liquid. Where the cells are adhered to a surface that is close to the centre of the tube, 5 mm from the wall of the tube, the exposure of these immobilised cells to heat is likely to be less even. In the bag method, where there is only a thin film of liquid between the cells on the substrate and the wall of the bag, the efficiency of heat transfer is likely to be greater than in the tube method. For this reason, the bag method is favoured and all the results discussed relate to the bag method.

Thermo-resistant streptococci varied in their susceptibility to heat, depending on the environment. The sensitivity to heat was not affected by adhesion to stainless steel in water. However, when the cells adhered in the presence of skim milk, the

sensitivity of *S. thermophilus* (H) decreased with a five-fold increase in the D-value at 60°C. Although test samples with adhered cells were rinsed, residual milk protein associated with the cell and the substrate may have protected the cells from heat. A seven-fold increase in the D-value at 60°C was also observed for planktonic cells in milk. This is consistent with the effect of organic material on microbial resistance to heat (Joslyn, 1983). The potential for thermo-resistant streptococci to survive heat treatment in a dairy manufacturing plant is therefore greater than the survival that may be expected for the organisms in other environments.

An increase in the resistance to heat of 12 and 24 h biofilms of thermo-resistant streptococci (grown in skim milk in a continuous flow laboratory reactor) compared with planktonic cells in water was observed. However, the D-values for 12 and 24 h biofilm cells at 60°C were less than those observed for cells adhered recently in the presence of skim milk. Possible explanations for this observation include stress on cells within the biofilm, the structure of the biofilm, chemical composition of the biofilm or alterations in the physiology of the cells in a growing biofilm. Clearly, there is no evidence that the formation of a biofilm of these organisms produces any materials that would protect these bacteria from heat, as the increased resistance to heat may be explained by the effect of milk protein. Increased resistance to heat of biofilm cells has been reported for other organisms. For *Listeria monocytogenes* (Frank and Koffi, 1990), increased resistance to heat was associated with the amount of growth on the substrate; for *Salmonella enteritidis* (Dhir and Dodd, 1995), increased resistance to heat was believed to be due to a change in the physiology of the cell induced by adhesion.

The presence of milk also affected the correlation values for the thermal death curves for the thermo-resistant streptococci. The correlation values were less for planktonic cells in milk, or cells adhered in the presence of milk, than for planktonic cells or adhered cells in water. This may reflect variations in the distribution of bacteria in

the milk with clumping around milk proteins resulting in the protection of cells and associated variation in the susceptibility to heat. This hypothesis requires further investigation.

The heat sensitivities of two strains of thermo-resistant streptococci obtained from a dairy manufacturing plant were compared. Strain 48 was more resistant than strain H, demonstrated by the greater D-values for planktonic cells of strain 48 compared with strain H. The most recent isolate was strain 48. This may reflect survival in a dairy manufacturing plant, with the most resistant strains like *S. thermophilus* (48) being the most likely to survive. Strain H may have lost some heat resistance through many passages in laboratory media. Strain 48 may have retained the ability to withstand high temperatures from its recent exposure in the dairy manufacturing plant. Enhanced resistance to heat following heat shock (52°C for 15 min) has been reported (Auffray *et al.,* 1995) and undoubtedly affects the survival of these bacteria in a dairy manufacturing plant, particularly where milk is heat treated (thermalised) to enable extended storage before pasteurisation.

The sensitivity to heat of the *S. thermophilus* used in this trial suggests that the temperature of > 70°C for 30 min, used in the routine cleaning of dairy manufacturing plant should be 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, including biofilm that 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.

The resistance of adhered and biofilm cells of thermo-resistant streptococci to chemical sanitisers was compared with the resistance of planktonic cells. The increase in resistance to sanitisers of biofilm cells compared with the planktonic cells of many

species is widely reported (Le Chevallier *et al.,* 1988; Yu and McFeters, 1994); however, there are no specific data for the thermo-resistant streptococci. In the present work, the resistance of recently adhered cells of both strains (H and 48) to sanitisers was similar to planktonic cells with the exception that strain H was more resistant to CTAB in the adhered form. Biofilm cells of both strains were more resistant to both sanitisers, with strain 48 being more resistant to chlorine than strain H. This increased resistance of biofilm cells to sanitisers may be due to organic material (*i.e.* milk protein) associated with the biofilm or protective mechanisms from other aspects associated with microbial colonisation of a surface (Wirtanen and Mattila-Sandholm, 1992b). With either sanitiser, biofilms of both strains of *S. thermophilus* survived the normal concentrations (200 ppm sodium hypochlorite, 25 ppm quaternary ammonium sanitisers) used in dairy manufacturing plants. The survival of any cells following treatment with sanitisers will allow the rapid regeneration of a biofilm, increasing contamination of the manufacturing plant and products.

To ensure that test conditions represented those that exist in an industrial situation, in the present study, the samples treated with sanitiser were rinsed to remove residual sanitiser before testing. No inactivator was used, as inactivators are not used in dairy manufacturing plant. In addition, sodium thiosulphate used to inactivate chlorine-based sanitisers was found to be toxic to thermo-resistant streptococci (data not shown). The growth medium used is likely to inactivate any sanitiser activity remaining following rinsing in a similar way to that of milk in a dairy manufacturing plant. The procedure used in this trial was similar to that used by Greene *et al.* (1993).

There were some concerns with the experimental methods used in this trial. Firstly, the biofilms may not have been representative of those in an industrial plant where there may be progressive accumulation of biofilm over several runs. Secondly, in a

manufacturing plant, sanitisers are used following cleaning whereas in this trial no cleaning chemicals were used. The need to ensure a reproducible colonisation of the substrate with high numbers of cells made the use of cleaning chemicals undesirable.

Future studies need to examine the effect of other sanitisers on biofilms of thermo-resistant streptococci. Hydrogen peroxide and peracetic acid sanitisers are being increasingly used in food manufacturing plants (Bird, 1997) and are effective on biofilms (Alasri *et al.,* 1992).

Enhanced resistance of biofilm cells of thermo-resistant streptococci to heat and chemical sanitisers is difficult to avoid. Therefore other methods to control biofilms are needed. Removal of the cells is one such method, reducing cell numbers and exposing the underlying cells making the remaining cells more susceptible to routine sanitising regimes.

To determine the effect of current industrial cleaning programmes on biofilms of thermo-resistant streptococci, a manufacturing plant was monitored using epifluorescence and conductance detection methods. Biofilms of thermo-resistant streptococci in the dairy manufacturing plant persisted after routine cleaning procedures. Although this plant was cleaned frequently (every 6 h), the total numbers of bacteria, detected by epifluorescence microscopy, before and after cleaning were similar, demonstrating that the routine cleaning procedure was not removing all of the bacteria from the substrate. Interestingly, the numbers of cells following each cleaning were similar *i.e.* there was no net increase in the numbers of bacteria over several manufacturing runs. The total numbers of bacteria for most sampling periods were 3-4 $\log_{10}$cells cm^{-2}, suggesting that the operating conditions, in particular the short operating time, prevented bacteria reaching the high levels (10^7 cells cm^{-2}) recorded by others (Bouman *et al.,* 1982).

The effects of different chemical treatments in removing thermo-resistant streptococci from stainless steel were compared in laboratory trials. These confirmed that acid and caustic chemicals failed to remove thermo-resistant streptococci from the surface of stainless steel. The investigation of different cleaning chemicals in removing recently adhered cells and 4 and 18 h biofilms of *S. thermophilus* (H) showed that treatments that affected proteins, particularly treatment with proteolytic enzymes, were most effective in reducing the total number of cells colonising the substrate. This corresponds with previous work described in this thesis demonstrating the importance of proteins associated with the cell surface in adhesion. Further enhancement of bacterial removal may be obtained by fine tuning these proteolytic treatments, with regard to concentration, exposure time and flow conditions.The concept of using enzymes for the removal of biofilms is not new. Polysaccharide-hydrolysing enzymes were found to be effective in removing biofilms of Staphylococci and Pseudomonads from steel and polypropylene (Johansen *et al.*, 1997).

The laboratory trials were followed with tests on biofilms in a pilot plant. The enhanced removal and successful inactivation of biofilms of thermo-resistant streptococci using a commercial proteolytic enzyme cleaner in the pilot-scale trial suggests that this may be a realistic alternative procedure for routine use in a milk pasteurising plant. No viable cells were detected (detection limit approximately 1 cell cm^{-2}) on the stainless steel following enzyme cleaning, although some adhered cells were still detected by epifluorescence microscopy. This suggests that the enzyme cleaner inactivates the cells. The pilot-scale work should be extended to demonstrate the effect of proteolytic enzyme cleaners in removing naturally occurring (rather than seeded) biofilms in dairy manufacturing plant during sequential manufacturing runs.

6.5 CONCLUSIONS

The method used to determine the response to heat of adhered cells influenced the results. Heat treatment of cells on stainless steel in a plastic bag produced D-values that were two-fold less than those obtained by treating these cells a test tube. This was explained by improved heat transfer using the bag method compared with the tube method.

Milk proteins increased the resistance of both planktonic and adhered cells to heat. Cells adhered to a stainless steel substrate in the presence of skim milk, but heat treated in water, were more resistant to heat than cells adhered in the presence of water. Biofilm cells grown in skim milk were more resistant to heat than planktonic cells in water. The resistance to heat of biofilm cells grown in skim milk was similar to that of cells that had recently adhered in the presence of skim milk although, in both situations, the heat treatment was done on washed surfaces in water.

Biofilms of thermo-resistant streptococci were more resistant to sodium hypochlorite and CTAB santisers than planktonic cells. Biofilms prepared in skim milk had the greatest resistance to these sanitisers and were resistant to sanitiser concentrations 5-50 times greater than those used in dairy manufacturing plant. This may be one explanation for the persistence of biofilms in dairy manufacturing plants.

Routine cleaning procedures in a dairy manufacturing plant were inadequate to remove and inactivate thermo-resistant streptococci colonising the stainless steel surface.

Proteolytic enzymes removed more biofilm cells of thermo-resistant streptococci than cleaning chemicals routinely used in dairy manufacturing plants.Trypsin reduced by

at least 100-fold the number of cells of thermo-resistant streptococci colonising a surface and was the best of the nine treatments tested in a laboratory trial. A commercial proteolytic enzyme cleaner, "Paradigm", also reduced by at least 100-fold the number of *S. thermophilus* colonising the substrate in laboratory trials. No viable cells remained colonising the substrate in a pilot plant following treatment with "Paradigm".

Chapter 7

FINAL DISCUSSION AND CONCLUSIONS

Biofilms are aggregations of microbial cells and their associated extracellular polymeric substances that are adhered to, growing on and multiplying on a surface (Bremer 1996). Biofilms are of concern in dairy manufacturing plants, as bacteria within biofilms are more difficult to eliminate than free living cells (Wirtanen and Mattila-Sandholm, 1992a; Matilla-Sandholm and Wirtanen, 1992; Mosteller and Bishop, 1993). Once established, biofilms can act as a source of contamination of product and other surfaces. Bacteria colonising the surface of milk processing equipment are increasingly implicated as a significant source of contamination of dairy products (Bouman *et al.,* 1981; Koutzayiotis, 1992; Flint *et al.,* 1997b). The most frequently reported biofilm problem in dairy manufacturing plants is the development of biofilms of thermo-resistant, thermophilic streptococci in pasteurisers and thermalisers, contaminating cheese milk and resulting in problems with cheese quality (Driessen and Bouman, 1979; Hup *et al.,* 1979, Driessen, *et al.,* 1984; Busscher *et al.,* 1990; Rademacher *et al.,* 1995; Limsowtin and Powell, 1996; Lee *et al.,* 1997). Although this problem is widely recognised, little is known of the factors leading to the development of biofilms of streptococci.

In this study, biofilm development was monitored in an industrial manufacturing plant. This is the first time that a modified Robbins device has been used to monitor the growth of streptococci on stainless steel surfaces in a manufacturing plant. The total and viable numbers of bacteria colonising the stainless steel were determined. A method was developed that enabled the use of the Malthus microbiological growth analyser as a sensitive tool to rapidly determine the numbers of viable cells on stainless steel samples from the modified Robbins device. This analysis confirmed

that routine caustic and acid cleaning was inadequate in removing and inactivating streptococci in biofilms. Isolates obtained from this monitoring trial were used to study the characteristics of these bacteria that promote the development of biofilms.

Isolates of streptococci causing problems in dairy manufacturing plant are often referred to as "*S. thermophilus* like" organisms. Confirming the identification of these bacteria was an important aspect of this thesis, providing a solid basis for subsequent investigations.

In the present trial, isolates from the dairy industry were identified based on culture growth, colony morphology, microscopic appearance, biochemical assays as well as molecular techniques. Culture growth and colony and cellular morphology were typical of streptococci. However, the biochemical assays revealed that the isolates fell into two groups with sufficient differences to suggest that these isolates belonged to two different species. The presence of two distinct groups was confirmed by screening the isolates using restriction endonuclease analysis, ribotyping and random amplified polymorphic DNA analysis. Polymerase chain reaction (PCR) amplification of specific sequences of DNA encoding rRNA in *S. thermophilus* and *S. bovis* (the two streptococci most commonly associated with the dairy industry and cattle respectively) revealed that isolates from one of these groups were *S. thermophilus*. Isolates in the other group could not be classified as either *S. thermophilus* or *S. bovis*. Using PCR, a variable region of the genome encoding 16S rRNA was amplified from those streptococci that could not be typed. The DNA sequence of this region was compared with published sequences and found to be different from those of other streptococci. A PCR primer and molecular probe were selected from this sequence to enable the rapid confirmation, through PCR and fluorescent *in situ* hybridisation, that all the isolates that were unable to be identified so far, were identical. DNA/DNA hybridisation between *S. thermophilus, S. bovis* and this new isolate demonstrated that the latter was sufficiently different to warrant

the label of a new species. This was supported by the DNA composition guanine + cytosine of the unknown isolates which was different from that of any published streptococci. This investigation formed the basis for the description of a new species, named *Streptococcus waiu* (waiu being the New Zealand Maori name for milk, reflecting the origin of these isolates). This species represented 24% of the isolates studied in this thesis.

In the formation of a biofilms, adhesion to the substrate is essential. Knowledge of the mechanisms of adhesion may assist in formulating methods to control the development of biofilms by designing processes to prevent adhesion or improving procedures to remove the organisms from the substrate. As there are no reports on the adhesion mechanisms of streptococci, this study aimed to study possible mechanisms. Although environmental factors, as well as the cell surface are all likely to affect adhesion, this investigation focused on properties of the cell surface.

Hydrophobicity of the cell surface, often associated with the adhesion of bacteria to substrates, was the first factor to be investigated. Characterisation of the isolates obtained for this study, showed that all isolates obtained from dairy manufacturing - either from the surface of the manufacturing plant, or from milk, or products from manufacturing plants experiencing contamination problems - were highly hydrophobic. In contrast, isolates from other sources produced mixed results. This suggested that hydrophobicity was important in the development of biofilms of streptococci. However, there was no correlation between hydrophobicity and the numbers of bacteria from standardised suspensions adhering to stainless steel. I postulate that hydrophobicity in some way predisposes cells to colonising stainless steel, with the more hydrophobic cells forming a stronger bond with the surface or being more likely to resist aqueous chemical cleaners than the more hydrophilic cells.

Another factor commonly associated with microbial adherence, cell surface charge, was investigated, with all cells demonstrating a variable net negative charge. Cells and substrates frequently possess a net negative charge which naturally repells cells from the substrate (Busscher and Weerkamp, 1987; Oliveira, 1992). This repulsion is overcome by stronger attractive forces (*e.g.* van der Waals' forces or hydrophobic interactions) to enable adhesion. It is therefore expected that cells with a lower negative charge or higher positive charge would be more likely to adhere. However, in the present trial, no correlation between negative or positive charges and the number of cells adhering was demonstrated.

The role of cell surface polysaccharides, often associated with adherence, particularly later in the adhesion process (Oliveira, 1992), was investigated by measuring the amount of polysaccharide produced by different isolates. No correlation was found between the amount of polysaccharide and the number of bacteria adhering to the stainless steel substrate. In addition, treatment of the cells with polysaccharide-degrading chemicals had no overall effect on adhesion.

The role of cell surface proteins was determined by treating the cells with chemicals that affected protein (trypsin and sodium dodecyl sulphate). These treatments reduced the number of cells adhering by approximately 100-fold, with similar results for all 11 strains using either treatment. These results suggested that proteins were important in the adhesion phase of biofilm development of streptococci. The proteolytic treatments also reduced the hydrophobicity of the cells; however, the importance of this in adhesion is doubtful because earlier results showed no direct relationship between hydrophobicity and adhesion. The cells were inactivated by the proteolytic treatments; however, tests on cells inactivated with formaldehyde, heat or ultraviolet light indicated that inactivation had little impact on the number of cells adhering. Therefore it appears that viable and non-viable cells adhere equally well.

Using sodium dodecyl sulphate polyacrylamide gel electrophoresis the effect of proteolytic treatments on the cellular proteins was shown to be the loss of a 55 kDa protein. This protein was therefore implicated in adhesion and was characterised by N-terminal sequencing, reveal a sequence resembling that of β-lactoglobulin. Treatment of the cells with antiserum to this protein reduced adhesion, providing further evidence for the involvement of this protein in adhesion. Immunolabelling enabled the protein on the cell surface to be visualised by transmission electron microscopy.

The involvement of proteins in adhesion has been reported for bacteria in mammalian systems (Jenkinson, 1994), and the adhesion of *Azospirillum brasilense* (nitrogen-fixing bacteria with plant growth promoting potential) to glass and polystyrene has been correlated with the protein concentration at the cell surface (Dufrêne *et al.,* 1996). β-Lactoglobulin appears to have an affinity with surfaces and it is believed to have a key role in fouling in dairy processing plants (Jeurnink *et al.,* 1996). The "adhesion protein" of streptococci is very similar to β-lactoglobulin. Future studies could develop a molecular probe for the "adhesion protein" of streptococci to investigate the wider involvement of this protein in the adhesion of other species.

The information on the importance of cell surface proteins in the adhesion process was used to develop improved methods of controlling biofilms of streptococci. Industry monitoring trials had already demonstrated that routine cleaning methods were unsatisfactory and this was confirmed in laboratory trials using biofilms grown in a specially designed laboratory reactor. The presence of milk proteins during the adhesion of streptococci increased the resistance of these cells to heat, represented by a five-fold increase in the D-value. There was some variation in the sensitivity of different strains of streptococci to chlorine and quaternary ammonium sanitisers; however, biofilms were all resistant to the normal strengths of both these sanitisers

used in routine cleaning of dairy manufacturing plants. This suggests that removal of the cells is likely to be more successful in controlling biofilms than inactivating them on the substrate and supports the approach taken in food manufacturing plants of thoroughly cleaning a plant before sanitising. However, the reduction in cells colonising the surface following caustic treatment was <10-fold and reductions following acid treatment were inconsistent. The greatest and most consistent reduction in biofilm cells (>100-fold) was obtained in the laboratory test system using trypsin. The success of an enzyme-based cleaner was supported by laboratory and pilot scale trials using a commercial proteolytic enzyme-based cleaner. This not only reduced the total number of adhering cells by >100-fold but also inactivated all detectable cells. If there are no viable cells remaining, the regeneration of biofilm will be reduced, enabling longer manufacturing runs before new cells colonise the surface.

The potential for enzyme based cleaners to control biofilms in dairy manufacturing plant requires further investigation in pilot scale and industrial sized manufacturing plant. It is difficult to reproduce accurately the conditions that exist in a dairy manufacturing plant, particularly the factors that accumulate during successive manufacturing runs. Many of the results reported in this thesis should be further explored on pilot scale or industrial scale plant operating in a continuous process/clean/process cycle with virtually no intercycle period. Fine tuning the temperature, exposure time and flow (turbulent flow was not achieved in the pilot-scale trial) may further improve this method of cleaning to control biofilms of streptococci. In addition, further investigations into the adhesion mechanisms of other microorganisms contaminating dairy manufacturing plant may lead to the wider use of enzyme-based cleaners.

Many other potential control methods need to be investigated. For example, in this thesis and elsewhere, the effectiveness of milk proteins in blocking the adhesion of

streptococci has been demonstrated (Bourassa *et al.*, 1996). If carefully managed, this could develop into a method of control. Further investigations into the importance of hydrophobicity in the overall development of biofilms of streptococci may lead to the design of cleaners (*e.g.* synthetic detergents) better able to remove the predominantly hydrophobic cells colonising the stainless steel substrate. If hydrophobic interactions are important in the development of a mature biofilm (suggested by the predominance of hydrophobic strains), altering the stainless steel surface (which is hydrophobic) may destabilise the biofilm, preventing heavy colonisation and assisting cleaning systems to remove biofilm.

In conclusion, the formation of biofilms of streptococci in dairy manufacturing plant in New Zealand has been shown to involve two species: *S. thermophilus* and *S. waiu*. Adhesion of these bacteria to the stainless steel substrate has been shown to be mediated by a protein associated with the cell surface. Trials demonstrated that proteolytic enzyme cleaners have the potential to improve present cleaning methods used in dairy manufacturing plant.

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Errata

Chapter 4

Isolates of thermo-resistant streptococci (both *S. thermophilus* and *S. waius*) were found in more than one manufacturing plant therefore it is unlikely that they were from a single clone.

Note that the standard deviations given in Figures 4.10 and 4.11 were calculated from two data points, therefore are not statistically valid. However they do give some idea of reproducibility of the data.