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Laboratory scale Clean-In-Place (CIP) studies on the effectiveness of different caustic and acid wash steps on the removal of dairy biofilms

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Abstract

A laboratory scale, bench top flow system was used to partially reproduce dairy plant conditions under which biofilms form and to quantify the effectiveness of caustic and acid wash steps in reducing the number of viable bacteria attached to stainless steel (SS) surfaces. Once bacteria attached to surfaces, a standard clean-in-place (CIP) regime (water rinse, 1% sodium hydroxide at 65 °C for 10 min, water rinse, 1.0% nitric acid at 65 °C for 10 min, water rinse) did not reproducibly ensure their removal. Standard CIP effectiveness was compared to alternative cleaning chemicals such as: caustic blends (Alkazolv 48, Ultrazolv 700, Concept C20, and Reflex B165); a caustic additive (Eliminator); acid blends (Nitroplus and Nitrobrite); and sanitizer (Perform). The addition of a caustic additive, Eliminator, enhanced biofilm removal compared to the standard CIP regime and further increases in cleaning efficiency occurred when nitric acid was substituted with Nitroplus. The combination of NaOH plus Eliminator and Nitroplus achieved a 3.8 log reduction in the number of cells recovered from the stainless steel surface. The incorporation of a sanitizer step into the CIP did not appear to enhance biofilm removal. This study has shown that the effectiveness of a "standard" CIP can possibly be enhanced through the testing and use of caustic and acid blends. There are many implications of these findings, including: the development of improved cleaning regimes and improved product quality, plant performance, and economic returns.

Keywords: Biofilms; CIP; Dairy plants; Caustic; Acid

1. Introduction

Bacterial contamination can adversely affect the quality, functionality and safety of products produced by the dairy industry. When contamination of dairy products occurs evidence suggests that biofilms on the surfaces of milk processing equipment are a major source (Koutzayiotis, 1992; Flint et al., 1997). Biofilms are aggregations of bacterial cells attached to and growing on a surface (Costerton and Stewart, 2001). Dairy biofilms invariably also contain significant milk residues, particularly protein and minerals such as calcium phosphate. Biofilms are not only a potential source of contamination, but can also increase corrosion rate, reduce heat transfer and increase fluid frictional resistance (Kumar and Anand, 1998). With current trends towards longer production runs, the use of complex equipment, the automation of plants, and increasingly stringent microbiological requirements, the contamination of dairy products due to the presence of bacterial biofilms has become a major concern to dairy manufacturers.

Biofilm control in dairy manufacturing plants (DMP) generally involves a process called Clean-In-Place (CIP), defined as the "cleaning of complete items of plant or pipeline circuits without dismantling or opening of the equipment and with little or no manual involvement on the part of the operator. The process involves the jetting or spraying of surfaces or circulation of cleaning solutions through the plant under conditions of increased turbulence and flow velocity" (Romney, 1990).

A feature of CIP regimes, evident in both industrial and laboratory scale system, is their variability in effectiveness in eliminating surface adherent bacteria (Austin and Bergeron, 1995; Faille et al., 2001; Dufour et al., 2004). This variability is not surprising as a large number of factors can influence CIP effectiveness including the: nature and age of the fouling layer; cleaning agent composition and concentration; cleaning time;

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cleaning agent temperature; degree of turbulence of the cleaning solution; and the characteristics of the surface being cleaned (Stewart and Seiberling, 1996; Changani et al., 1997; Lelievre et al., 2001, 2002a,b; Boulange-Petermann et al., 2004). Further, many processing lines will contain areas prone to fouling such as dead ends, joints, valves and gaskets (Austin and Bergeron, 1995; Wong, 1998) and surfaces whose chemistry, surface topography (pit and crack formation) and ease of cleaning changes with use (Storgards et al., 1999).

As cleaning effectiveness is dependent on both product and processing plant specific variables, the optimal CIP regime varies between DMPs and over time within a given plant. Unfortunately the optimisation of CIP effectiveness is difficult in DMP due to their large size, and their focus on production rate, product quality and safety.

In the dairy industry CIP systems generally involve the sequential use of caustic (sodium hydroxide) and acid (nitric acid) wash steps, chemicals originally selected for their ability to remove organic (proteins and fats) and inorganic (calcium–phosphate and other minerals) fouling layers, in some instances a sanitizer step is also applied (Chisti, 1999).

The most common and aggressive caustic cleaner is sodium hydroxide (NaOH), which is typically used in 1–5% concentrations for plate-type and tubular heat exchangers, and other heavily soiled surfaces and 1–2 % for general use (Flint et al., 1997). The primary role of the caustic (alkali) wash step is the removal of proteins and carbohydrates (Chisti, 1999). Increasing the effectiveness of the caustic step may reduce the amount of nitric acid required and the need to use a sanitizer. To enhance cleaning effectiveness caustic blends and caustic additives have been developed which contain surfactants, emulsifying agents, chelating compounds and complexing agents. The primary role of the acid step is to remove the mineral scale left on the plant surfaces after exposure to the caustic cleaning chemical. The acid step also aids in the removal of traces of alkaline product from equipment surfaces, enhances draining and drying and provides bacteriostatic conditions that delay the growth of any remaining micro-organisms (White and Rabe, 1970; Stewart and Seiberling, 1996). The most common acid used in the dairy industry is nitric acid and as with caustic chemicals, acid blends have been formulated which may contain other acids or surfactants. Traditionally chlorine (sodium hypochlorite) based sanitizers have been used, however, a wide variety of sanitizers including quaternary ammonium compounds, anionic acids, iodophores and chlorine based compounds are currently in use or being evaluated for use in CIP regimes (Alasri et al., 1992, 1993; Rossoni and Gaylarde, 2000; Joseph et al., 2001; Parkar et al., 2004).

Using combinations of sodium hydroxide, nitric acid, caustic blends, nitric acid blends, caustic additives, and the peracetic acid/hydrogen peroxide sanitizer, Perform (Orica, Chemnet), this study, used a recently developed flow loop reactor system (Dufour et al., 2004) to compare the relative effectiveness of full or partial CIP regimes against dairy biofilms developed on stainless steel (SS) surfaces under conditions typical of those encountered in dairy manufacturing plants.

2. Materials and methods

2.1. Experimental system used for dairy biofilm development

A continuous flow bioreactor, described by Dufour et al. (2004), which was comprised of a milk reservoir a re-circulating test loop, a reactor, and a waste reservoir was used to develop mixed species bacterial biofilms on surfaces of SS tubes. Skim milk powder (Fonterra Clandeboye Powder Plant, Temuka, New Zealand, batch No. MH 1575 BL14 51108) was reconstituted to 10% total solids to replicate the concentration of milk in the milk treatment section of the Fonterra Clandeboye Powder Plant. For each experiment, 21 L of milk was prepared and held at 4 °C before being passed through a heat exchanger to reach 55 °C immediately prior to entering the reactor. The re-circulating test loop, which re-circulated milk from the reactor, contained a number of 304 grade, 2B finish SS tubes (6.5 mm diameter; 15 mm length; 3.06 cm^2 internal surface area) that had been cleaned using a CIP regime, consisting of a 10 min wash with NaOH (1% w/v, 65 °C) (BDH), a 5 min cold distilled water rinse, a 10 min wash with Nitric Acid (1% w/v, 65 °C) (Orica Chemnet, NZ), a 5 min cold distilled water rinse, a 30 min soak in detergent (Decon 90, Biolab Scientific), and a distilled water rinse. The SS tubes were inserted into sections of silicon tubing (size 3/16 in., Degania Silicon, Israel) in triplicate groups, allowing for easy sampling and aseptic removal. Pieces of larger silicon tubing were used to overlap the joins between the 3/16 in. tubing, producing a firm seal. The completed bioreactor system was sterilized by autoclaving. Milk was pumped (Cole Palmer, Masterflex, peristaltic pump head model 77200-52, Biolab Scientific) around the re-circulatory test loop at a velocity of 1.5 m s^{-1}. Milk was pumped into the reactor (300 ml), and out to waste at 600 ml h^{-1} in order to achieve a dilution or turnover rate of 2 h^{-1}. As the doubling time of the micro-organisms in the milk was 0.52 h this flow rate limited the growth of micro-organisms in the reactor. To develop a biofilm on the SS tubes, milk was circulated through the re-circulating test loop and reactor system for 18 h.

2.2. Effectiveness of a caustic and acid clean-in-place (CIP) regime

SS tubes exposed to milk for 18 h were rinsed by the circulation of distilled water through the test loop and three SS tubes were removed and designated the “no CIP” control tubes. The remaining SS were exposed to the following CIP regime, which was designated as our “standard” CIP. A 1 L volume of NaOH solution (1%) was circulated through the tubing for 10 min at 1.5 m s^{-1}, followed by an intermediate water rinse (5 min total; 2 min single passage at 1.0 m s^{-1}, 3 min re-circulating at 1.5 m s^{-1}) and a wash with nitric acid (1%) for 10 min at 1.5 m s^{-1}. A final water rinse (5 min total) completed the CIP. All cleaning solutions were kept at a constant temperature of 65 °C using a water bath. After each water rinse, triplicate SS tubes were removed and the bacteria on the surface of the SS tubes removed using a swabbing technique previously described in

Dufour et al. (2004). The resulting suspension was diluted in peptone (0.1%, Tryptone water, Merck) and plated in triplicate on standard plate count agar (SPCA, Difco) plates, overlaid with SPCA (4 ml). Plates were incubated at 50 °C for 24 h. Representative bacterial colonies were Gram and spore (modified Wertz) stained and examined microscopically.

2.3. Effectiveness of alternative caustic cleaning chemicals

To determine if modifying the caustic acid regime enhanced the effectiveness of the CIP, four alternative caustic cleaning chemicals: Alkazolv 48 (1% w/v); Concept C20 (1% w/v); Ultrazolv 700 (1% w/v) and Reflux B165 (0.65% v/v) (Table 1. Orica Chemnet) were tested and compared against each other and the standard CIP regime which used NaOH (1% w/v). Each CIP consisted of one of the caustics followed by nitric acid with water rinses as per the general CIP procedure described above. This experiment was carried out over four trials, due to the time needed for the processing of the samples. To enable inter-trial comparison some of the treatments were repeated over several trials and samples that were not exposed to cleaning (no CIP) as well as samples that were only exposed to the standard NaOH regime were included in every trial to check reproducibility of results. For each trial, SS tubes were fouled with milk and rinsed with distilled water as previously described. Three SS tubes were subsequently removed (no CIP control) and the tubes remaining in the re-circulating test loop were divided into five sets, each containing three SS tubes. Each set of SS tubes underwent a separate caustic wash and intermediate water rinse, and was then rejoined with the other sets of three tubes for the nitric acid wash and final water rinse. The concentrations used were recommended by Orica and were selected to reflect, as closely as possible the caustic concentration (1%) used in the milk treatment section of a milk powder plant. Following CIP treatment, the five sets of three SS tubes were disconnected and processed using the previously described techniques. To improve the reproducibility of the biofilms developed on the SS tubes, the first and last SS tubes in the test loop system were not used. To reduce possible experimental artefacts due to processing, the order in which the SS tubes in the loop were removed for processing and the order in which the different CIP regimes were tested were varied from trial to trial.

2.4. Effectiveness of caustic cleaning chemicals plus additive

To investigate if the incorporation of a caustic additive increased the effectiveness of biofilm removal, Eliminator (Table 1, Orica Chemnet, NZ) was added at a concentration of 1.25% v/v to the five different caustic cleaning chemicals used above and incorporated into the CIP regime. This study was carried out over four trials and contained repeated treatments and controls to check reproducibility. Each trial was carried out as outlined above.

2.5. Effectiveness of alternative acid cleaning chemicals

To determine the effectiveness of alternative acid cleaning chemicals on biofilm removal, three different acid cleaning chemicals were tested at 65 °C for 10 min against an 18 h biofilm. The cleaning chemicals used were; Nitric acid, Nitrobrite, and Nitroplus (Table 1, Orica, Chemnet), all at a concentration of 1% (w/v). The SS tubes were fouled with milk and rinsed with water as previously described. Three SS tubes were subsequently removed prior to cleaning (no CIP control) and the remaining tubes in the test loop underwent a caustic wash of NaOH (1%) plus Eliminator (1.25%) at 65 °C, followed by an intermediate water rinse and three SS tubes were removed as a caustic treatment control. The remaining SS tubes were divided into three sets of three SS tubes. Each set of SS tubes underwent a different acid wash for 10 min at 65 °C and a final water rinse, before being processed using the methods described earlier.

2.6. Effectiveness of sanitizer

To investigate the effectiveness of the sanitizer Perform (Table 1, Orica, Chemnet) in reducing the number of cells attached to SS, an 18 h biofilm was developed and the SS tubes rinsed with distilled water, as previously described. Three SS tubes were subsequently removed prior to cleaning (no CIP control) and the remaining tubes in the test loop were divided into four sets of three SS tubes. Each set of SS tubes underwent a different CIP. The four CIP treatments used were: 1. Standard CIP; 2. Standard CIP with Perform (0.35% v/v, 20 °C); 3. Optimized CIP (based on results from Sections 2.3–2.5); NaOH (1%)+Eliminator (1.25%) (65 °C) and

Table 1
Description of the CIP blends and additives used in the study

Name	Product	Description
Alkazolv 48	Caustic blend	Contains stable surfactants for improved surface wetting plus chelating and sequestering agents to keep metal ions in solution during the cleaning cycle
Concept 20	Caustic blend	A low viscosity caustic which gives good assistance with the rate of soil removal and promotes good rinsing of soiled solution from the plant
Reflux	Caustic blend	Contains complexing agents and a surfactant system to emulsify and sequester soils
Ultrazolv 700	Caustic blend	Contains a low foam surfactant system, degreasing aids and calcium phosphate chelating acids
Eliminator	Caustic additive	Contains chelating and sequestering agents and surface active wetting agents
Nitroplus	Nitric blend	Contains nitric acid plus surfactants
Nitrobrite	Nitric blend	Contains nitric and phosphoric acid
Perform	Sanitizer	A stabilized formulation of peracetic acid in hydrogen peroxide

Information was obtained from product information sheets supplied by Orica Chemnet (www.orica-chemnet.com).

Nitroplus (1%, 65 °C); 4. Optimized CIP with Perform (0.35%, 20 °C). The caustic and acid cleaning chemicals were circulated around the test loop according to the general CIP procedure and then for the 2nd and 4th CIP regimes (see above); sanitizer was circulated around the test loop for 3 min at room temperature. Following each CIP, the tubing was disconnected and the SS tubes were processed using the techniques described earlier.

2.7. SEM examination

In order to visualize the biofilms formed on the surface of the SS coupons, as well as the effects of the CIP regime on the biofilm, CIP treated and untreated fouled SS surfaces were examined by scanning electron microscopy (SEM). To facilitate sample preparation, SS coupons rather than tubes were used in this experiment. The SS coupons (304 grade, 2B finish, surface area of 7.3 cm^2) were polished using 1200 grit sandpaper (Norton), and passivated using a modified CIP regime outlined previously for the SS tubes. The coupons were inserted into plastic tubing (Para Rubber, 15 mm), which was sterilized by autoclaving. After autoclaving, milk was added to the reactor system and circulated through the tubing containing the SS coupons for 18 h. After 18 h the system was stopped, the tubing was disconnected, and the coupons divided into three sets of six SS coupons; one set did not receive a CIP regime (no CIP control); another set received the caustic cycle of the optimized CIP; and the final set received the caustic and acid cycles of the optimized CIP. Three of the coupons from each set of 6 had total bacterial numbers estimated by swabbing and plating (using the standard method previously described) in order to enable a comparison with cell numbers obtained in previous experiments using SS tubes. The remaining three coupons from each set were put into labelled vials (30 mL) containing 2.5% glutaraldehyde in 0.1 M cacodylate buffer (10 mL) to undergo primary fixation (2 h). Fixed samples were subjected to washes in cacodylate buffer (0.1 M) and exposed to osium tetroxide (2.1%) in cacodylate buffer (0.1 M) (2 h), washed again in cacodylate buffer (0.1 M) (three 15 min washes) and finally dehydrated in a graded series of ethanol (25%, 15 min; 50%, 15 min; 70%, 15 min; 85%, 15 min; 95%, 15 min; 100%, 30 min; 100%, 30 min; 100%, 30 min). The samples were then critical-point dried (Balzers 030 CPD critical point dryer), mounted on aluminium stubs with double sided carbon tape and silver paint, and coated with gold and palladium in a Bio Rad Sputter coater for two minutes. Mounted samples were viewed with a Cambridge 360 Scanning Electron Microscope.

2.8. Statistical analysis

Analysis of variance (ANOVA) were conducted on the log transformed data to determine if any significant differences ($p < 0.05$) lay between the treatments and Tukey's test was used to distinguish where these differences lay. Assumptions of equal variance amongst the treatments and normality (normal distribution) were also checked. In the Eliminator and caustic additive trial, independent sample *T*-tests (Welch *T*-test) were carried out to find significant differences ($p < 0.01$) on each relevant sample pair because variance inequality between different treatments meant ANOVA was not the appropriate method.

3. Results

3.1. Biofilm formation

Examination by SEM of the SS coupons surfaces after exposure to milk for 18 h revealed the presence of both single cells and large colonies of bacteria (Fig. 1A–C). The bacteria

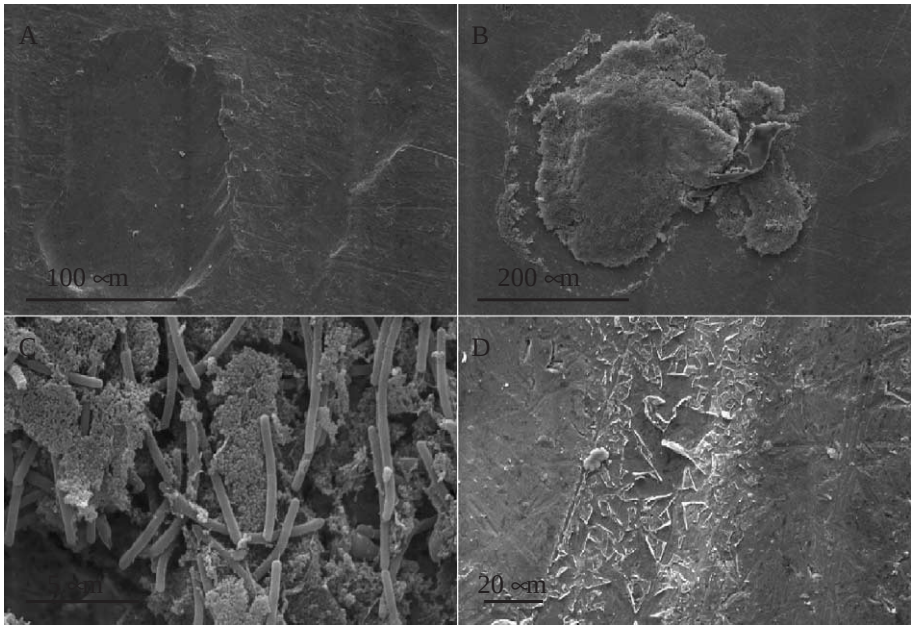

Fig. 1. SEM images of fouled SS surfaces before (A, B and C) and after (D) the "optimized" CIP.

were usually associated with debris and presumably protein and carbohydrate material. On occasions large clumps of cells were visible (Fig. 1B–C). The fixation and dehydration process used to prepare the sample caused a slight shrinkage as the biofilm pulled away at the edge (Fig. 1B). The number of viable cells recovered from the surface of the SS tubes which were capable of growth on SPCA at 50 °C ranged from 1.6×10^4 to 6.8×10^5 cfu cm^{-2}, over all the trials. The predominant bacterial colonies recovered were comprised of Gram-positive spore forming bacilli. Gram positive cocci, presumably thermo-resistant Streptococci, were also isolated.

3.2. Effectiveness of standard Clean-in-Place (CIP) procedure

The effectiveness of our standard CIP regime (using NaOH and nitric acid) was determined against an 18 h biofilm (Fig. 2). The caustic wash achieved a 1.8 log reduction ($p < 0.05$) in the number of cells recovered from the SS tubes when compared to the "no CIP" control tubes. The acid wash appeared to have little additional impact on cell numbers.

3.3. Effectiveness of alternative caustic cleaning chemicals

The effectiveness of four caustic blends as well as NaOH on biofilm removal was tested over four trials containing duplicates of the treatments to check reproducibility of the results obtained (data not shown). The number of bacteria recovered after each trial and each treatment were standardized against the "no CIP" control tubes (Fig. 3). The standardized caustics, when compared to no CIP control SS tubes, achieved between 0.75 to 2 log reductions ($p < 0.05$) in the number of cells recovered from the biofilm. However, for the standardized caustics there was no significant difference in the number of cells recovered from the biofilm after treatment with either NaOH, Alkazolv, Concept or Reflux, with only Ultrazolv being significantly ($p < 0.05$) more effective than NaOH at reducing cell numbers. Ultrazolv, however, was not significantly better at reducing bacterial numbers compared to Reflux. The relatively low level of cell reduction achieved by the five

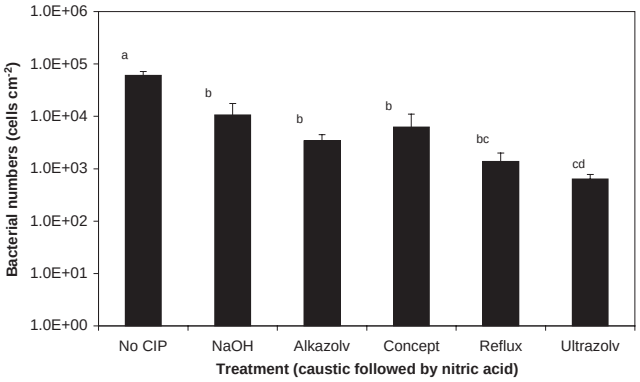

Fig. 3. The mean standardized number of cells recovered from SS tubes, fouled with a standard 18 h biofilm, before cleaning, and after exposure to a caustic wash followed by a nitric acid wash. Numbers are expressed as the mean of three replicate samples ± the standard deviation of the mean. Different letters signify significant differences.

caustic treatments and the small variation in their effectiveness suggest that for practical purposes the alternative caustics were not "significantly" better that the standard caustic.

3.4. Caustic additive effect

The effectiveness of incorporating an additive (Eliminator) in association with caustic cleaning chemicals on biofilm removal was tested over four trials incorporating duplicates of the treatments to check reproducibility of the results (data not shown). The bacterial cell numbers for each Eliminator trial and each treatment were standardized against the "no CIP" control tubes and compared with the standardized NaOH bacterial numbers (Fig. 4). The caustic plus Eliminator and nitric acid CIP were shown to be significantly more effective at reducing biofilm than the standard CIP (NaOH and nitric acid). To detect any significant differences between the 5 caustic plus Eliminator CIP regimes independent sample *T*-tests (Welch *T*-test) were conducted on the caustic plus Eliminator treated tubes data. This test was used instead of ANOVA because although the average number of cells remaining after the caustic Eliminator treatments were fairly similar, there was a

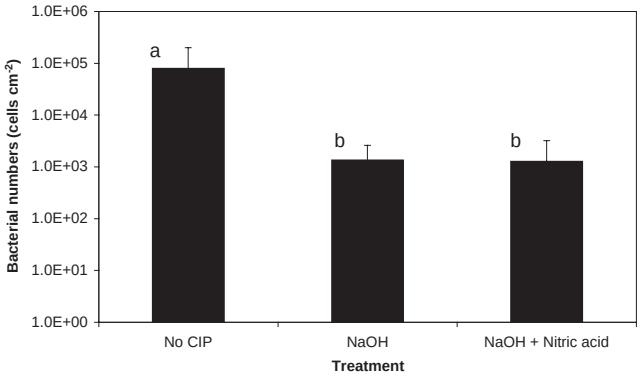

Fig. 2. Mean number of cells recovered from SS tubes, fouled with a standard 18 h biofilm, before cleaning, and after exposure to either the caustic step or the caustic and acid steps of a CIP regime. Numbers are expressed as the mean of 3 replicate samples ± the standard deviation of the mean. Different letters signify significant differences.

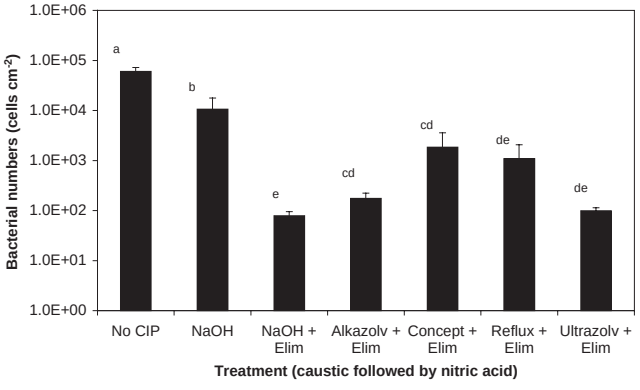

Fig. 4. The mean standardized number of cells recovered from SS tubes, fouled with a standard 18 h biofilm, before cleaning, and after exposure to a caustic plus Eliminator wash step followed by a nitric acid wash. Numbers are expressed as the mean of three replicate samples ± the standard deviation of the mean. Different letters signify significant differences.

Table 2
Means and standard deviations of numbers of cells remaining after the caustic, Eliminator and nitric acid treatments

Treatment	Cell numbers ($\log_{10}$ cm^{-2})
NaOH Eliminator	1.85±0.21
Ultrazolv Eliminator	1.98±0.13
Alkazolv Eliminator	2.15±0.31
Reflux Eliminator	2.49±0.86
Concept Eliminator	2.53±0.84

large variation in the standard deviations (Table 2), and variance inequality meant ANOVA could not be used. The most effective combination at removing the biofilm was the NaOH plus Eliminator CIP, which achieved almost a 3 log reduction in bacterial numbers recovered from the biofilm compared to untreated SS tubes, and just over a 2 log reduction in bacterial numbers compared to the effectiveness of the standard CIP. The NaOH plus Eliminator combination was significantly better ($p>0.01$) than the Alkazolv plus Eliminator CIP, and the Concept plus Eliminator CIP (Table 3), but not significantly better than the Ultrazolv plus Eliminator CIP, or the Reflux plus Eliminator CIP.

3.5. Effectiveness of alternative acid cleaning chemicals

The effectiveness of two alternative acid cleaning chemicals (Nitroplus and Nitrobrite) in removing attached cells from SS surfaces was compared to the effectiveness of Nitric acid. A standard 18 h biofilm was exposed to three different CIP regimes involving NaOH plus Eliminator, followed by one of the three acid cleaning chemicals (Fig. 5). Nitroplus was significantly ($p<0.05$) the most effective acid-cleaning chemical at reducing the biofilm when used in combination with NaOH and Eliminator. This combination (the "optimised CIP" regime) showed a reduction in bacterial numbers of nearly four logs compared to numbers recovered from the "no CIP" control SS tubes.

3.6. Effectiveness of sanitizer

The effect of Perform sanitizer in further removing attached cells from SS surfaces was investigated using a standard 18 h biofilm exposed to different CIP regimes (standard CIP and optimized CIP), either with or without sanitizer (Fig. 6). Results for both the standard CIP and the optimized CIP indicated that the addition of a sanitizer step did not significantly ($p>0.05$) reduce bacterial numbers. The surface of the SS coupons after caustic step of the optimized CIP was

Table 3
T-test results for caustic, Eliminator and nitric acid treatments

Treatment	Ultrazolv Elim	Alkazolv Elim	Reflux Elim	Concept Elim
NaOH Elim	0.056	0.003*	0.076	0.008*
	Ultrazolv Elim	0.044	0.138	0.023
		Alkazolv Elim	0.320	0.118
			Reflux Elim	0.904

* signifies significant differences ($p<0.01$).

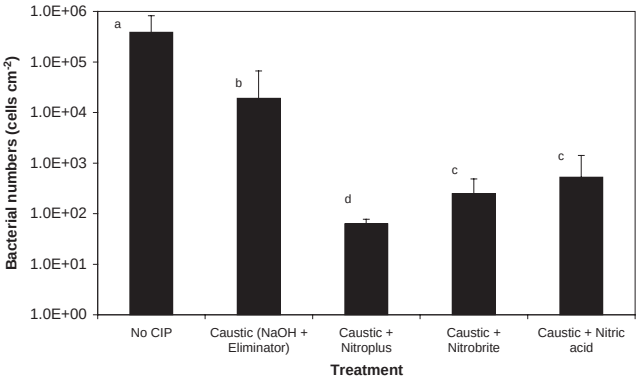

Fig. 5. Mean number of cells recovered from stainless SS, fouled with a standard 18 h biofilm, before cleaning, and after exposure to either a caustic wash only, caustic and Nitroplus washes, caustic and Nitrobrite washes or caustic and Nitric acid washes. Numbers are expressed as the mean of 3 replicate samples ± the standard deviation of the means. Different letters signify significant differences.

imaged using SEM (Fig. 1D). Clumps of bacteria were not detected, and the few bacteria, which were visible, occurred as single cells. Further, material, presumably protein, that had covered the fouled coupons as a virtually uniform layer (Fig. 1A) showed areas of flaking and large patches of bare SS where the film had been completely removed (Fig. 1D).

4. Discussion

The effectiveness of CIP regimes in removing gram-positive bacteria known to form biofilms in dairy plants, such as *Streptococcus thermophilus* and *Bacillus* species, has only relatively recently received attention (Flint et al., 1997, 1999; Lindsay et al., 2002; Parkar et al., 2003, 2004). Although chemical companies tend to push "new" and "innovative" cleaning chemicals, there is little published research on the relative effectiveness of different chemical cleaners and an apparent lack of techniques that can simply and effectively determine their effectiveness. Further, as the effectiveness of a CIP regime is affected by many plant and process specific variables it is important to "tailor-make" a cleaning regime to

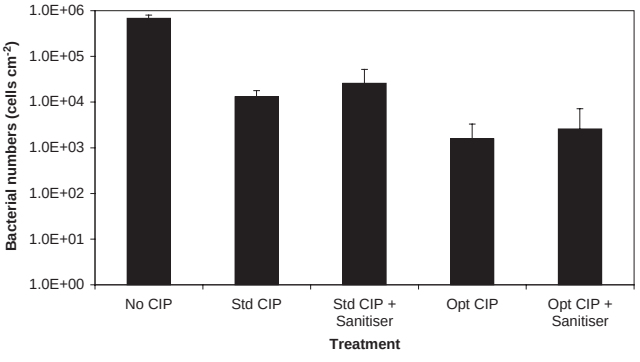

Fig. 6. Mean number of cells recovered from SS tubes, fouled with a standard 18 h biofilm, before cleaning, and after exposure to a standard (NaOH and nitric acid) (Std) or optimized (NaOH/Eliminator and Nitroplus) (Opt) CIP alone, or combined with Perform sanitizer. Numbers are expressed as the mean of 3 replicate samples ± the standard deviation of the means.

suit the conditions of the plant, and to therefore have a simple and reliable way to ensure optimum cleaning is achieved.

We used a flow loop reactor system to monitor the fouling and cleaning of dairy biofilms under conditions which simulated a pre-heat section of milk powder plant in terms of milk flow velocity (1.5 m s^{-1}), temperature (55 °C), milk contact surface and contaminating micro-organisms. This system was inexpensive to construct, easy to run, and permitted testing of multiple SS tubes in a single reactor run which in turn allowed the effectiveness of the complete CIP system, as well as its individual components, to be tested. Throughout this study skim milk powder sourced from the Fonterra Clandeboye DMP was used in order to maintain similarities between the plant conditions and the laboratory system. Further, to reduce any differences between trials and to help produce a consistent and reproducible biofilms on the surface of the SS tubes all the skim milk powder came from a single batch. This approach appears to have been successful as the standard 18 h biofilms used throughout the trials contained relatively similar numbers of cells (within 1.6 logs), ranging from 1.6×10^4 to 6.8×10^5 cfu cm^{-2}. There is some debate in the literature about the time required to develop consistent, relevant biofilms. For example, it has been reported that a minimum of 48 h is required to develop a "true" biofilm on SS using a meat soup test medium (Wirtanen and Mattila-Sandholm, 1992). In contrast, in systems similar to the one used in the current studies mature biofilms have been reported to occur after 12 to 18 h (Flint et al., 2001; Parkar et al., 2003; Sharma et al., 2003). SEM analysis of the fouled surfaces in this study revealed that the bacteria formed large complex colonies of cells (Fig. 1) and were therefore presumably growing on the SS surface rather than simply attaching from the bulk phase. The examination of SS surfaces by SEM after swabbing revealed a total absence of any large clumps of cells with only the occasional single cell detected. This data suggested that the swabbing technique employed was removing the vast majority of the attached cells. This result is similar to those from other studies carried out in this laboratory which have reported that while swabbing cannot ensure the removal of all attached cells, recoveries in excess of 99.98% are achievable (Bremer et al., 2001; Dufour et al., 2004).

Using a cleaning simulator, Dunsmore et al. (1981) showed that alkaline and acidic cleaning chemicals were required for effective cleaning but that for the cleaning chemicals to be effective they had to be applied under turbulent conditions on the surface. The CIP regime used in this study was modelled on cleaning regimes commonly used in dairy manufacturing plants and incorporated both an alkaline wash to remove the alkaline-soluble (proteins and fats) deposits and an acid wash to remove the acid-soluble (calcium phosphate and other minerals) components of the milk deposit (Chisti, 1999; Dunsmore, 1981).

In assessing the effectiveness of the standard CIP against an 18 h biofilm (Fig. 2) it was found that while a mean 2 log reduction in bacterial numbers was achieved after the caustic treatment (NaOH, 1%), no further significant reduction occurred after the acid treatment (Nitric acid, 1%). Over the course of 10 trials, each with triplicate samples (Figs. 2,3,4 and 6) the standard CIP was largely ineffective. This finding was consistent with previous studies which have reported large variations in the ability of NaOH to consistently remove dairy biofilms (Flint et al., 1999; Dufour et al., 2004). However, these results differ from those reported by Parkar et al. (2004), who report that a CIP regime comprising of 2% NaOH at 75 °C for 30 min, 15 min distilled water rinse at ambient and 1.8% nitric acid at 75 °C for 30 min completely killed cells in a mature biofilm of *Bacillus flavothermus* containing 7×10^7 cells cm^{-2}. Reasons for this difference in CIP effectiveness may be related to the higher concentrations of NaOH and nitric acid used, the difference in treatment temperature and duration and the fact that Parkar et al. (2004) used a single species biofilm rather than a mixed consortium as in the current study.

To determine if the addition of sequestering agents and surfactants to the basic caustic step could reduce the variability and increase the effectiveness of the CIP, a variety of CIP regimes involving different caustic blends with nitric acid were tested and compared to the standard CIP regime and to untreated samples. Results showed that there was a 1–2 log reduction in bacterial numbers recovered from the treated SS tubes when compared to the SS tubes that did not undergo a CIP treatment, but only a small to negligible reduction, in bacterial numbers recovered when compared to the standard CIP regime (Fig. 3). In addition there was no improvement in the variability of counts obtained between replicate trials. This inherent variability in the system meant that significant differences could not be accurately determined. The addition of Eliminator significantly reduced the number of cells remaining on the SS tubes after cleaning (Fig. 4). This finding is supported by studies which reported that the addition of surfactants and sequestering agents improved the overall performance of the caustic wash step by a factor of ten and that the addition enhanced the removal of both fat and protein soiling and minimized hard water effects (Stewart and Seiberling, 1996; Changani et al., 1997). In the current study the "best" caustic plus Eliminator mix was determined to be NaOH plus Eliminator. However, as there are no significant differences between the effectiveness of NaOH plus Eliminator, Alkazolv plus Eliminator or Concept plus Eliminator, NaOH plus Eliminator was chosen as the "best" caustic based, in part, on its lower cost as well as its performance.

Alternative acids to nitric acid were investigated to determine if a further improvement in biofilm reduction could be made. Two acids blends were tested (Nitrioplus and Nitrobrite) alongside nitric acid, using the same concentration and temperature of nitric acid so comparisons under the same conditions could be made. Nitroplus was significantly more effective at reducing cell numbers than the other two acids. Nitroplus treatment achieved a 3.8 log reduced in the number of bacterial cells recovered compared to bacterial numbers recovered from SS tubes that had not undergone a CIP. Nitroplus also reduced by an additional 1 log the number of bacterial cells recovered compared to numbers recovered after treatment with caustic followed by nitric acid. There is little published data regarding the use of acid blends to improve CIP

effectiveness. A reason for this lack of evidence may be that removal of the biofilm is believed to be predominantly achieved by the caustic step (Dunsmore, 1981; Chisti, 1999) and therefore research efforts have been mainly directed towards improving this step. The current study has shown that the acid step can also be enhanced.

Once the best performing basic CIP regime had been determined experiments were conducted to assess the impact of sanitizer on further reducing the biofilm. The sanitizer experiment failed to show a reduction in cell numbers after treatment of the stainless steel tubes with Perform (0.35%), in fact, a slight (but not significant) increase in cell numbers was seen. Eginton et al. (1998) found that in all cases following disinfection treatment, attached cells were more easily removed from the test surfaces. Therefore in our trials perhaps the sanitizer step resulted in the cells being more easily removed and separated and therefore resulted in a higher viable count being recorded. In general while sanitizers such as chlorine, iodophores, anionic acids, peroxyacetic acid, and quaternary ammonium compounds have proven to be effective against planktonic bacteria their effectiveness against bacterial biofilms is variable (Mosteller and Bishop, 1993; Rossoni and Gaylarde, 2000; Bremer et al., 2002).

In our experiments only single fouling and cleaning cycles were examined and it is reasonable to postulate that the effectiveness of the cleaning regimes studied may vary over a number of fouling and cleaning cycles. The small scale of the test system and its ability to accommodate the removal and analysis of SS tubes of time make this system an attractive option to use to study changes in cleaning effectiveness over time. The experimental technique used in this study has been shown to be reproducible and to have the potential to provide valuable insights into the effectiveness and limitations of CIP regimes and the individual steps within CIP regimes. Further, this research has shown that the removal of bacterial biofilms on surfaces in DMP can be enhanced by the use of caustic and nitric additives.

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