Characterization of the Microbial Flora in Disinfecting Footbaths with Hypochlorite

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ABSTRACT

Change or disinfection of footwear are measures to prevent cross contamination between areas with low and high hygienic levels in the food industry. The efficacy of disinfecting footwear is not well documented. Samples of used disinfectant and from swabbing of corners after draining were taken from disinfecting footbaths containing chlorine in four Norwegian cheese factories. Bacteria were present in 9 of 12 footbaths and more positive samples were found from swab samples than from used disinfectant. The microbial flora in footbaths varied between the dairies. In two dairies, the flora was dominated by Pseudomonas spp. and Acinetobacter spp., respectively. In the third dairy, both Bacillus spp. and Staphylococcus spp. were present and in the fourth dairy, the flora was diverse (Acinetobacter sp., Enterococcus faecalis, Klebsiella pneumoniae, and Bacillus sp.). The strains were not resistant to the recommended user concentration of chlorine in bactericidal suspension or surface tests. The degree of attachment to plastic varied between strains and species and bacteria attached to surfaces were in general more resistant than suspended bacteria. The results of the survey indicated that disinfecting footbaths containing chlorine may act as contamination sources in food factories and should not be used without regular hygienic monitoring.

A high hygienic level is crucial for the production of safe, high quality food. The raw materials may be contaminated with pathogens and spoilage microorganisms via air, food contact surfaces, and food handlers through the food production chain. Disinfectants are often used on a daily basis to reduce microbial contamination on food contact surfaces and in the food production environments. They may also be used to disinfect footwear, equipment, and personnel that enter areas with higher hygienic level. By contrast to most other disinfection routines in food factories, disinfection of footwear often involves the use of the same disinfection solution over a long period of time. In laboratory tests for evaluation of the efficiency of disinfectants, microorganisms are exposed to high concentrations of the disinfectant for a short time interval (e.g., 5 min) before neutralization and determination of the lethal effect. Although a disinfectant may be effective in this context it is not necessarily suitable for use in footbaths. There is little documentation about the hygienic level in footbaths and which microorganisms that may survive during long-time exposure to disinfectants.

A survey on the use of disinfecting footbaths in Norwegian dairies showed that chlorine compounds were most frequently used, followed by products based on amphoteric tensides and alkyl amino acetate (28). Bacteria were present in 75% of the footbaths tested and none of the disinfectants prevented bacterial survival in all footbaths. Sporeformers and gram-negative bacteria dominated the bacterial flora in footbaths containing amphoteric tensides or alkyl amino acetate (14). Serratia marcescens, which was isolated from footbaths in all six dairies using amphoteric tensides or alkyl amino acetate, showed survived exposure to user concentration of amphoteric tensides in a bactericidal test, but not alkyl amino acetate (14). This demonstrated that for some tenside-based disinfectants, long-time exposure to user concentrations may select for resistant gram-negative bacteria. Therefore, the hygienic level of the footbaths should be monitored on a regular basis.

A few reports describing the microbial flora surviving on surfaces in food-processing lines after cleaning and disinfection with hypochlorite have been published. Mettler and Carpentier (18) showed that the microflora on the floor in a dairy factory cleaned with a chlorinated alkaline chemical (no disinfection step) was dominated by Pseudomonas spp. and Staphylococcus spp. The flora in seafood factories after cleaning and disinfection with hypochlorite was dominated by yeasts and Pseudomonas spp. (4). Kocuria sp., Brevibacterium sp., and Staphylococcus sp. are important resident microorganisms surviving in cheese factories and Kocuria may protect the more sensitive S. sciuri against hypochlorite in a binary biofilm (15).

As far as we know, information about the microbial flora in disinfecting footbaths with hypochlorite has not been published previously. This article presents the results from a survey of disinfecting footbaths in four Norwegian dairies. A diverse flora with spoilage and pathogenic bacteria with a range of susceptibilities to hypochlorite was identified. It is hypothesized that survival of bacteria in disinfecting footbaths is a result of physiological resistance such as biofilm formation.

MATERIALS AND METHODS

Isolation of strains. Four cheese factories participated in the survey. They used hypochlorite concentrations recommended by
the disinfectant manufacturers (500 to 1,000 µg ml⁻¹) and changed disinfectants in the footbaths every day (dairies A, B, and D) or every second day (dairy C). Dairy A alternated between hypochlorite and a disinfectant based on cationic tensides and acetate acid. The footbaths were made of plastic material and in addition to the disinfectant solution, they contained mats of plastic (dairies A, B, and C) or foam rubber (dairy D). Disinfecting footbaths were sampled at the end of the production day using the following sampling methods: (i) disinfectant solution from the footbath (2 to 3 ml) was collected in a tube, (ii) 1 to 2 drops of disinfectant from the footbath were diluted in 3 ml tryptone soy (TS) broth (Oxoid, Hampshire, UK), and (iii) after draining, a corner of the footbath was swabbed with a cotton swab and the bacteria isolated suspended in TS broth. The samples were taken from footbaths that were visually clean and located in rooms with a temperature of 20 to 25°C. A total number of 12 footbaths were included in the study. After arrival at the laboratory, a volume of 10 µl of each sample was spread on plate count agar (PCA; Difco, Sparks, Md.) and incubated at 20°C. The plates were visually inspected for growth after 5 days of incubation. Colonies with different appearance on the agar plates were randomly selected and re-streaked on PCA resulting in a total of 22 isolates. The strains were stored at −80°C in TS broth with 15% glycerol.

Identification of bacteria. Total DNA was isolated from 3-ml cultures grown overnight in TS broth with the Easy-DNA kit (Invitrogen Corporation, Carlsbad, Calif.). More than 1,450 bp of the 16S rRNA genes (16S rDNA) were amplified by PCR using the primers K1r+KR2 (23). The 16S rDNA PCR products were sequenced on an ABI Prism 3100 Genetic Analyser, using the Big Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer Applied Biosystems, Foster City, Calif.) and primers previously described (13). Database search was performed with BLASTN 2.1.2 (2).

Surface attachment. Bacterial isolates identified as non-sporeformers were further characterized. If more than one isolate of the same species were isolated from the same footbath only one representative isolate were further studied. The bacteria were grown in shaking cultures overnight in TS broth at 25°C and inoculated (1:100) in TS broth (45 ml) to a final cell concentration of about 10⁸ cells ml⁻¹. Polyacetal coupons (75 by 22 by 1 mm; POM C Ertacetal Natur, Vink Plast, Kolbotn, Norway) were placed vertically in the broth and the culture incubated for 18 h at 25°C with shaking (200 rpm). The coupons were removed from the culture and gently washed for 2 min in peptone water to remove loosely adherent cells before determination of the number of attached cells by sonication and plating. Bacteria were detached by sonication for 20 min at 30°C (40 kHz, 130 W; Bransonic 3510, Branson Ultrasonic B.V., Soest, The Netherlands). After sonication, the number of CFU was determined by serial dilution of attached cells by sonication and plating. Bacteria were detached by sonication for 20 min at 30°C (40 kHz, 130 W; Bransonic 3510, Branson Ultrasonic B.V., Soest, The Netherlands). After sonication, the number of CFU was determined by serial dilution of attached cells by sonication and plating. Bacteria were detached by sonication for 20 min at 30°C (40 kHz, 130 W; Bransonic 3510, Branson Ultrasonic B.V., Soest, The Netherlands). After sonication, the number of CFU was determined by serial dilution of attached cells by sonication and plating. Bacteria were detached by sonication for 20 min at 30°C (40 kHz, 130 W; Bransonic 3510, Branson Ultrasonic B.V., Soest, The Netherlands). After sonication, the number of CFU was determined by serial dilution of attached cells by sonication and plating. Bacteria were detached by sonication for 20 min at 30°C (40 kHz, 130 W; Bransonic 3510, Branson Ultrasonic B.V., Soest, The Netherlands). After sonication, the number of CFU was determined by serial dilution of attached cells by sonication and plating.

Susceptibility determination. The susceptibility of suspended cells was tested by exposing the bacteria (approximately 10⁷ cells ml⁻¹) to 50 and 500 µg ml⁻¹ hypochlorite (Klorin, Lilleborg, Oslo, Norway) in 1% skim milk for 5 min at 20°C before neutralization of the disinfectant and determination of CFU. The test conditions were based on recommendations given in the European Standard method for disinfectants used in the dairy industry (3). The recommended in-use concentration for surface disinfection in the food industry was 0.5 to 1.0 % of the concentrate, corresponding to 500 to 1,000 µg ml⁻¹ hypochlorite. The bacterial test suspension was made by diluting overnight cultures in TS broth at 30°C in peptone water and further 1:1 in 10% skim milk to a final cell concentration of about 10⁸ cells ml⁻¹. A 1-ml test suspension was inoculated in 4 ml disinfectant made up in sterile distilled water. The cells (approximately 10⁷ cells ml⁻¹) were exposed to the disinfectant (50 and 500 µg ml⁻¹ hypochlorite) for 5 min before neutralization by tenfold dilution in Dey Engley neutralizing broth (Difco). Control samples were treated the same way, but without disinfectant. The CFU was determined by plate spreading on TS agar and incubation at 30°C for 5 days. The logarithmic reduction in cell count was calculated by subtracting the log (CFU ml⁻¹ of the test mixture after the action of the disinfectant) from log (CFU ml⁻¹ of the test mixture without disinfectant). Susceptibility to 50 and 500 g ml⁻¹ hypochlorite was also tested for three selected strains pre-grown in 1% skim milk.

Susceptibility of attached cells was determined by exposing coupons with attached cells to 50 and 500 µg ml⁻¹ hypochlorite in 1% skim milk for 5 min at 20°C, transfer of coupons to neutralizing broth, detachment of cells, and determination of CFU. The coupons were placed in glass tubes containing either 45 ml hypochlorite solution or distilled water. After exposure for 5 min (20°C) the coupons were transferred to tubes with 45 ml Dey Engley neutralizing broth. The number of attached cells was determined by sonication and plating as described above. The experiments were performed three times on different days and with freshly prepared solutions.

Statistical analysis. Minitab (19) was used to calculate statistical significance of differences between means (two-sample Student’s t test and one-way ANOVA) and regression analysis. Log values of cell numbers were used for all statistical tests.

RESULTS

Microbial flora in footbaths. Four footbaths were sampled at dairies A and C, and two footbaths at dairies B and D. In total, bacteria were isolated from 9 of the 12 footbaths tested. Bacteria were isolated from two and three footbaths in dairies A and C, respectively. In dairies B and D, both footbaths contained cultivable bacteria. Bacteria were isolated from both disinfectant solution and from swab samples, with the exception of dairy B, where only swab samples contained viable bacteria. A total number of 22 isolates were chosen for further study. All isolates appeared dissimilar on nutrient agar (size, color, or morphology) or they were isolated from different footbaths. An overview of the results from the isolation of strains is given in Table 1.

Identification by 16S rDNA analysis showed that the microbial flora differed between the dairies. None of the bacterial species identified were found in more than one dairy. The flora in footbaths from dairies A and D were dominated by species within the genera Pseudomonas and Acinetobacter, respectively. The flora in dairy B appeared more diverse, as all four strains isolated were of different


### TABLE 1. Identification of strains isolated from disinfecting footbaths

<table>
<thead>
<tr>
<th>Dairy</th>
<th>Footbath no.</th>
<th>Isolation method</th>
<th>Strain</th>
<th>Identity (sequence similarity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A×</td>
<td>2</td>
<td>i</td>
<td>A2</td>
<td>Pseudomonas pseudoalcaligenes (100)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>i</td>
<td>A3</td>
<td>P. putida (100)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>iii</td>
<td>A4</td>
<td>P. fulva (100)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>i</td>
<td>A5</td>
<td>P. putida (99.5)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>iii</td>
<td>A6</td>
<td>P. putida (99.6)</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>iii</td>
<td>B1</td>
<td>Acinetobacter baumannii (99.9)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>iii</td>
<td>B2</td>
<td>Enterococcus faecalis (99.8)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>iii</td>
<td>B3</td>
<td>Klebsiella pneumoniae (100)</td>
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<td></td>
<td>5</td>
<td>iii</td>
<td>B4</td>
<td>Bacillus fusiformis (99.9)</td>
</tr>
<tr>
<td>C</td>
<td>6</td>
<td>ii</td>
<td>C1</td>
<td>Brevibacterium casei (99.6)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>ii</td>
<td>C2</td>
<td>Bacillus mycoides (100)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>iii</td>
<td>C5</td>
<td>Staphylococcus warneri (100)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>iii</td>
<td>C6</td>
<td>S. aureus (99.9)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>ii</td>
<td>C4</td>
<td>Bacillus silvenstris (100)</td>
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<tr>
<td></td>
<td>8</td>
<td>ii</td>
<td>C3</td>
<td>B. mycoides (99.9)</td>
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<tr>
<td></td>
<td>8</td>
<td>iii</td>
<td>C7</td>
<td>Staphylococcus pasteuri (100)</td>
</tr>
<tr>
<td>D</td>
<td>9</td>
<td>iii</td>
<td>D1</td>
<td>Acinetobacter lwoffi (99.7)</td>
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<td></td>
<td>10</td>
<td>ii</td>
<td>D3</td>
<td>A. lwoffi (100)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>ii</td>
<td>D4</td>
<td>A. calcoaceticus (100)</td>
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<td></td>
<td>10</td>
<td>iii</td>
<td>D5</td>
<td>A. johnsonii (99.6)</td>
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<td>A. calcoaceticus (100)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>i</td>
<td>D7</td>
<td>A. calcoaceticus (100)</td>
</tr>
</tbody>
</table>

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*a* i, disinfection solution; ii, 1 to 2 drops of disinfectant in 3 ml of TS broth; iii, swab sample of footbath after draining.

*b* Species that gave the highest sequence match by Blast 2.1.2 search in GenBank.

*c* Dairy A alternated daily between an acid-disinfectant–containing cationic tenside and hypochlorite.

genera. Gram-positive cocci and sporeformers dominated in dairy C. Three different species of Staphylococcus were identified in this dairy, including the pathogen S. aureus.

**Hypochlorite sensitivity.** Exposure of the strains from disinfecting footbaths to the lowest recommended in-use concentration of Klorin (0.5% of concentrate, 500 μg ml⁻¹ hypochlorite) resulted in more than 5 log reduction in CFU in the suspension test. The susceptibility of sporeformers was not tested due to their well known intrinsic resistance.

For bacteria attached to surfaces the detection limit of the test varied, depending on the total number of bacteria attached to the plates. More than 4 log reduction in cell count was obtained for most strains exposed to 500 μg ml⁻¹ hypochlorite. A. lwoffi D3 and B. casei C1 survived (log reduction between 2.9 and 3.3) in two of three tests. A. calcoaceticus D4 survived (2.9 log reduction) in one out of three tests. To be able to differentiate between strains, further testing was performed with a concentration of one tenth of the recommended in-use concentration. Figure 1 shows log reduction of bacteria exposed to 50 μg ml⁻¹ hypochlorite in suspension and attached to surfaces. On average, bacteria attached to surfaces were more resistant to hypochlorite than bacteria in suspension ($P = 0.007$) as the mean reduction was 0.5 log reduction for attached cells and

![FIGURE 1. Log reduction of cells exposed to 1/10 of the recommended in-use concentration of hypochlorite. Cells in suspension (white bars) or attached to polycetal coupons (grey bars) were exposed to 50 μg ml⁻¹ hypochlorite for 5 min at 20°C. The mean standard error was 0.4 and 0.3 for the suspension and the surface test, respectively.](http://example.com/figure1.png)
FIGURE 2. Total number of cells attached to polyacetal coupons (grey bars) and total number of cells in surrounding medium (white bars) after incubation for 18 h at 25°C in TS broth. Mean log CFU of two replicates with standard error shown. Also, the percentage of cells attached is calculated for each strain.

1.8 in the suspension test. However, no significant correlation was found between the two methods (correlation coefficient of −0.067) indicating that differences found between strains in suspension test would not necessarily reflect what was observed in the surface test.

The susceptibility to 50 μg ml⁻¹ and 500 μg ml⁻¹ hypochlorite in suspension was tested for three strains isolated from used disinfectant in footbaths (B. casei C1, A. calcoaceticus D4, and P. pseudoalcaligenes A2) pregrown in 1% skim milk. The susceptibility level was similar to what was found using TS broth for precultivation (data not shown).

Surface attachment. The ability of the strains to attach to polyacetal is shown in Figure 2. The fraction of the bacterial cells attaching to the polyacetal coupons varied between different genera (P < 0.05). Pseudomonas spp. attached in high numbers (mean log CFU, 7.9; mean fraction attached, 1.4%) followed by Staphylococcus spp. (mean log CFU, 7.1; mean fraction attached, 0.04%) and Acinetobacter spp. (mean log CFU, 6.8; mean fraction attached, 0.1%). As Klebsiella, Enterococcus, and Brevibacterium were only represented by one strain, it was not possible to compare the genera statistically. The K. pneumonia B2 strain attached in high numbers, comparable with Pseudomonas strains. Brevibacterium casei C1 was among the strains with lowest attachment.

DISCUSSION

The microflora in disinfecting footbaths with chlorine has, as far as we know, never been reported in the scientific literature previously. The present study shows that the flora in footbaths with hypochlorite was diverse and varied between the dairies. This could be due to differences in the contamination flora, disinfectant concentrations applied, or load of organic material neutralizing the hypochlorite. In two dairies, the flora was dominated by Pseudomonas spp. and Acinetobacter spp., respectively. In the third dairy both Bacillus spp. and Staphylococcus spp. were present and in the fourth dairy it was a diverse flora (Acinetobacter sp., Enterococcus faecalis, Klebsiella pneumoniae, and Bacillus sp.). Most species isolated were common spoilage bacteria and likely contamination sources are raw milk or the environment (soil, water). The finding of food pathogens (S. aureus) and opportunistic pathogens (K. pneumoniae, A. baumannii) indicated that disinfecting footbaths may not be effective actions to prevent spread of pathogens in the factory.

The bacteria from footbaths are among the genera most frequently isolated from food processing environments (11) and have been reported to be important spoilage organisms in cheese (7, 10, 16, 22). Both Bacillus spp. and Staphylococcus spp. were isolated from dairy C. It is likely that Bacillus were present as spores, which have a high intrinsic resistance to disinfectants. Bacillus cereus and other pathogenic Bacillus species that survive pasteurization may represent a potential problem for the dairy industry, but none of the species of Bacillus identified in this study were pathogenic. Staphylococcus spp. are frequently isolated from biofilms in food-processing environments (11, 29) and likely contamination sources are raw milk or personnel. S. aureus is of the most common pathogens responsible for contagious mastitis in ruminants and the ability to form biofilm is considered to be a major virulence factor (27). Survival of endemic strains of S. aureus after cleaning procedures has been explained by production of polysaccharide resulting in physiological resistance to chlorine (6).

Pseudomonas spp., which dominated in one dairy, are common spoilage bacteria in raw and pasteurized milk and the contamination sources for the latter may be air or filling equipment (9). Pseudomonas spp. may survive cleaning and disinfecting routines in food factories and this has been attributed to high intrinsic resistance to disinfectants, low nutrient requirements, and adherence to surfaces (4). Acinetobacter spp. are frequently isolated from raw materials (such as raw milk), food-processing equipment and food products, but seldom identified as a major spoilage bacterium (12, 26). Acinetobacter is an emerging opportunistic pathogen and it has been speculated that food is the contamination source in hospital-acquired infections (5). However, strains from food differ from clinical strains in a num-
ber of ways and transmission from insufficiently cleaned surfaces to patients is a more likely explanation than food borne infection in clinical environments (8, 10). Acinetobacter is reported to be susceptible to disinfection and survival on inert surfaces has been explained by tolerance to drying (4, 8).

Unfortunately, the concentration of active chlorine in used disinfectant was not measured and survival could potentially be explained by neutralization of disinfectant by organic matter. However, this cannot be the whole explanation as the footbaths were visually clean and the disinfectant changed frequently. The results indicated that the active concentration of chlorine in dairies B and C was sufficient to prevent survival of vegetative bacteria in solution, but not on the inner surface of the footbath. It is well known that bacteria growing in biofilm, and in multispecies biofilm in particular, may survive high concentrations of chlorine (21). The explanation for this phenomenon is that hypochlorite is neutralized by reaction with biofilm components (25). In dairy B no bacteria were isolated from the disinfecting solution and most likely the bacteria were protected against hypochlorite by dirt neutralizing the hypochlorite or biofilm growth. Similarly, all Staphylococcus strains (dairy C) were isolated from swab samples indicating that they were present on the surface of the footbaths and not surviving in the disinfectant solution. High susceptibility in the suspension test and relatively large differences in susceptibility of suspended and attached bacteria were common for all vegetative bacteria from dairy C. With the exception of the staphylococci and one Brevibacterium strain, all other isolates from dairy C were sporeformers which have a high intrinsic resistance. In contrast to dairies B and C, the relatively uniform flora in dairies A and D indicated a strong selective pressure. Pseudomonas spp. and Acinetobacter spp. were isolated from used disinfectant solution without immediate neutralization, indicating long time survival in hypochlorite. The chlorine tolerance and degree of attachment varied within the genera Pseudomonas and Acinetobacter. Three of the Pseudomonas strains differed from the other isolates in the degree of attachment, but surprisingly attachment did not necessarily lead to higher survival during exposure to chlorine. Since the susceptibility to antibacterial agents may be dependent on pregrowth medium, we tested susceptibility after cultivation in 1% skim milk. However, this did not affect susceptibility to chlorine in our study. In conclusion, the results from laboratory model studies were not able to give a good explanation for the survival of Pseudomonas and Acinetobacter in the footbaths.

The results obtained from the bactericidal tests were not unique, as numerous factors influence susceptibility to disinfection and there are several examples of bacteria surviving practical disinfection being susceptible to in-use concentrations of disinfectants in laboratory tests (17, 20). Pregrowth conditions may affect tolerance to antibacterials, but cultivating bacteria in skim milk did not have a significant impact on the susceptibility. Several investigations have demonstrated that bacteria isolated after disinfection are relatively more resistant than corresponding laboratory or sporadic strains (1, 6, 24). In initial experiments we tested culture collection strains from each genus for their susceptibility to chlorine, but (with the exception of Acinetobacter), there were no indications of higher intrinsic resistance of footbath strains (not shown). It is therefore not likely that the isolates survived in footbaths due to acquisition of resistance or that they were selected for their high intrinsic resistance. Most publications on bacteria surviving exposure to chlorine are related to the concentrations used in swimming pools and water distribution systems and are less than one-tenth of the lowest concentration tested in the present study. The dairies involved in the study used the concentration intended for disinfection of equipment (500 to 1,000 μg ml⁻¹) and none of the isolates survived this concentration in the bactericidal test. A few isolates survived occasionally, but a longer exposure time would probably result in total elimination in the laboratory test. As also showed by other studies, bacteria attached to surfaces were, with a few exceptions, more resistant than suspended bacteria. The results from the laboratory model system could, however, not support that attachment alone resulted in survival during exposure to in-use concentrations of chlorine.

The results showed clearly that it is not always relevant to use suspension tests to predict survival on surfaces. It is well known that attached bacteria may survive in higher numbers, but the effect of attachment on the susceptibility varied highly between different strains and species. Strains with different susceptibility in suspension test could have similar survival in the surface test and vice versa.

This investigation showed that a broad spectrum of bacteria, including spoilage and pathogenic bacteria may survive in disinfecting footbaths with chlorine. The bacteria varied in their susceptibility to chlorine and degree of attachment to surfaces. We have earlier reported that disinfectant-resistant Serratia marcescens survives in footbaths with the amphoteric tenside Tego—a completely different disinfectant. In conclusion, disinfecting footbaths should not be used without careful hygienic monitoring.

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