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NORDFOOD
Sanitation in dairies

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ABSTRACT

The project started with an industry survey of commercial cleaning and sanitation agents used in the Nordic countries. Based on this survey a list of different types of priorized cleaning agents and disinfectants were included. The variation in testing procedures and scientific data acheived for these agents raised the motivation to prepare a harmonized testing procedure for agents used in the dairy industry. Parameters used in foam cleaning in open systems and cleaning-in-place (= CIP) procedures in closed systems were chosen to design the sanitation experiments in pilot scale. Disinfectant testing both in suspensions and on surfaces were included in the experiments.

Traditional cultivation was selected as a routine method. The cultivation method assessed the viable bacteria, which were able to form colonies on agar. The staining procedure was developed and the swabbing technique using novel swabbing detergents was evaluated in pilot and process scale. The cultivation results in comparison to microscopy showed that sampling from a surface was improved when the swabs were moistened with a detergent solution containing surfactants or enzymes and abrasives. The image analysis of preparations stained with acridine orange assessed biofilm and soil left on the surface. The microscopical technique using DNA stains and metabolic indicators enabled the distinction of dead and viable cells left on the surfaces. Furthermore, methods such as impedance, contact agar, agar moulding and ATP were used in the assessments.

The ***testing of*** six ***disinfectants*** selected by the dairy partners, Valio Ltd. and Norwegian Dairies, were performed using tests proposed by CEN (prEN 1040 and prEN 1276). The microbes used were standard test organisms as well as spoilage bacteria, pathogens and spores of concern in dairies. The prEN 1040 did not differentiate between the six disinfectants, whereas high levels of interfering substances e.g. skimmed milk and hard water used in prEN 1276 reduced the efficiency of the majority of the disinfectants to variable degrees. *Staphylococcus aureus* and *Pseudomonas aeruginosa* were the most resistant to all disinfectants tested. *Salmonella infantis* was the most difficult to kill of the four ”industrial” strains. Spores of *Bacillus cereus* and *Bacillus thuringiensis* were reduced by less than 1 log unit in the suspension test. The testing of disinfectants using 80% of the lowest recommended concentrations of the agents differentiated well between their

efficiency at the described test conditions given in prEN 1276. These results showed that the alcohol-based IPA 300 was the most efficient of the agents tested. More stringent tests are, however, clearly needed to simulate the conditions of practical application. Results in surface tests showed that *Pseudomonas fragi* cells dried on surfaces was more resistant to the disinfectants than were cells in suspension.

The results obtained in the experiments on ***cleaning of closed systems*** showed that treatment with alkaline cleaning agents containing chelating agent in combination with potassium hydroxide was more efficient in removing soil and biofilm than the alkaline agent containing only sodium hydroxide. The suitability of a cleaning agent was dependent on the consistency of the soil and the type of bacteria to be removed from the surface and the surface material. The results of the microscopic and impedance assessments after the surfaces were swabbed showed that some biofilm remained on the surface after all the cleaning procedures were performed.

The results from the tests using ***low pressure cleaning of open systems*** showed that treatment with a strong alkaline cleaning agent containing hypochlorite was more efficient in removing biofilm than a milder alkaline agent designed for non-processing surfaces. The level of the bacteria on unsoiled surfaces was lower than on presoiled surfaces. The results, however, indicated that the low pressure application system was not effective in removing all the biofilm unless the foaming agent itself was very effective. Therefore, the foaming agent must remain in contact with the surface for a sufficient length of time without drying. The efficiency of the foaming agent was dependent on its ability both to remove biofouled layers from the working surface and to kill the bacteria in the biofilm.

The aim of ***fogging or disinfection testing in industrial scale*** was to study the efficiency of the disinfection on the exposed surfaces at different places in the room. Fogging may be defined as ”chemical disinfection by means of automatic spraying of the disinfectant into a closed room”. Controlled experiments with fogging were carried out at two cheese producing dairies. Neither of the fogging trials showed clear reduction of the microbial load. The experiments at both dairies clearly demonstrated the need for thorough control and follow up of fogging. Critical points were the quality and amount of the fog, the disinfectant concentration and the rinsing. Insufficient rinsing only diluted the disinfectant and the disinfectant was not removed totally. The use of fogging also increased the humidity in the room, which may increase corrosion and damage on electrical equipment. Conclusion of the fogging experiment was that the effect was questionable, especially in critical points and also in dry rooms.

PREFACE

This research work in the NORDFOOD project P93156 ”Sanitation in dairies” was carried out at VTT Biotechnology and Food Research, the Norwegian Food Research Institute Matforsk and the Swedish Institute for Food and Biotechnology SIK during the years 1994 - 1996. The industrial partners involved in the project were Valio Ltd. from Finland and Norwegian Dairies. Dr. Tiina Mattila-Sandholm, VTT Biotechnology and Food Research, coordinated the project and Dr. Seppo Sivelä, Valio Ltd., chaired the project board meetings. Dr. Per Mården from SIK functioned as the coordinator of the Hygiene projects in the NORDFOOD programme. The project group and the steering group have met eight times during the project. Financial support was provided by the Nordic Industrial Fund, the Technology Development Centre TEKES in Finland and Nærforsk in Norway.

The experiments were focused on monitoring methods in sanitation of open and closed systems. The sanitation of closed systems was mainly carried out by VTT and SIK. The research at Matforsk lead by Dr. Gunhild Sundheim focused on disinfectant testing both in suspension and on surfaces. The work at VTT, which focused on development on test methods for and sampling of biofilms on dairy equipment surfaces, was planned by Dr. Tiina Mattila-Sandholm and Dr. Gun Wirtanen. Experiments with detergent solutions were performed in cooperation with the industrial partner Orion Diagnostica Ltd. in the TEKES project P467/94 ”Development of biofilm diagnostics”.

Information of results obtained in this project was delivered to an extended audience at the international symposium ”Future prospects of biofouling and biocides” in Helsinki on 13th - 14th of June 1996. The symposium was arranged by VTT Biotechnology and Food Research in cooperation with the University of Helsinki. The scientific programme was planned by Dr. Tiina Mattila-Sandholm and Prof. Mirja Salkinoja-Salonen. Furthermore, Dr. Gun Wirtanen and Dr. Laura Raaska took part in the organizing committee of the Symposium. The aim of this symposium was to provide knowledge of biofouling problems and hygiene in both food and nonfood industrial sectors. The session on detection methods presented the state-of-the-art in microscopic techniques as well as in molecular biology. The newest research results on biocides and disinfectants were presented in the session on elimination of biofouling by representatives of manufacturers and scientists. The abstract and summary of the results acheived in this project have also been translated into Swedish, Norwegian and Finnish (Appendices 3 - 5).

CONTENTS

1 INTRODUCTION

Development of biofouling causes many problems in industry. Biofouling consist of both microorganisms and their extracellular products usually polysaccharides and glycoproteins as well as organic soil. In course of time the extracellular polysaccharides and increase in microbial numbers lead to the development of a multilayered biofilm. If the cleaning and sanitation is inadequate the products will be contaminated. Once developed, biofilm is difficult to remove completely. Parameters affecting the cleaning of surfaces are chemical agents, mechanical forces, temperature and duration of the cleaning procedure. In the food industry the cleanliness of surfaces, instruments and equipment affects the safety and quality of the products.

In closed systems the biofilm leads to cleanability problems, particularly if the equipment surfaces are rough or in cases where there are irregularities in the design of the equipment bends, seals, crevices, dead ends and grooves. The mechanical forces of the cleaning processes in closed systems are proportional to the flow rate of the cleaning fluid. It is not enough to kill the microorganisms, the remaining matrix must also be removed. If it is not removed, the remaining matrix will provide excellent opportunity for the fast establishment of a new biofilm. It is thus of utmost importance to prevent the formation of biofilm and in cases where biofilm is formed to remove both the microorganisms and other organic material in the biofilm effectively from the surface.

Biofilm formation also increases the resistance to chemicals and antibiotics due to changes in cell physiology when the cells are attached to surfaces. Furthermore, disinfectants tend to leave the slime intact when no mechanical treatment is involved in the sanitation. Disinfection in the dairy industry is carried out using both hot water and chemical disinfection. The selection of the best disinfectants to be used in practical situations can be difficult. It depends e.g. on the microorganisms to be killed, the type of food processed, the surface material, the hardness of the water and the disinfection method e.g. spraying on open surfaces. Today most disinfectants are tested using suspension tests, but little is known of the relevance of the results to industrial problems where biofilms or surface attached bacteria are the reality. Endospores are more resistant to disinfectants than vegetative cells, and like the latter shows inter- and intra-species variation in resistance to heat and chemical compounds can be noticed. Therefore spores of two *Bacillus* strains were included in the disinfectant testing.

In industrial environment disinfectants can be applied by fogging. Fogging can be defined as ”chemical disinfection by means of automatic spraying of the disinfectant into a closed room”. This technique was introduced to the Norwegian dairy industry in the early nineties. Today about ten dairies, all of them producing cheese, are using the method. Most of them

started up with fogging in 1994 or later. Exact knowledge of fogging and its effect is, however, limited. The two main goals of the experminents in this project were to learn more about how fogging works, to investigate and if possible to document the efficiency of disinfectants on microbes applied using fogging in the dairy environment.

1.1 PROJECT PLAN

The experiments performed in the project have been summarized in Figure 1. These experiments focused on sanitation in both open and closed systems as well as on development and selection of microbial detection methods and sampling methods for dairy purposes. The methods were evaluated in pilot scale studies using the rigs at VTT Biotechnology and Food Research.

The detection methods used were conventional cultivation, cultivation using detergent based swabbing solutions, image analysis linked to epifluoresence microscopy, viability of organisms using metabolic indicators, contact agar, agar moulding, ATP and impedance. Cleaning agents and disinfectants used in Nordic dairies were listed both in the begining and at the end of the project (lists with agents used in Finland 1993-94 and in Norway 1996 are given in Appendices 1 - 2). The study of cleaning regimes in open systems started using surfaces with biofilms of *Pseudomonas fragi* and *Listeria monocytogenes.* The sanitation was performed using low pressure cleaning with foam cleaners. Representatives from both Matforsk and SIK took part in cooperation trials on cleaning of open systems on the test rig at VTT in the autumn 1995. The sanitation of closed systems was mainly carried out at the test rigs at VTT and SIK. A representative from Matforsk participated in the pilot scale experiments performed during spring 1996.

The research at Matforsk was focused on disinfectant testing both in suspension and on surfaces. As a first step suspension tests in Draft European Standard Methods (prEN 1040 and prEN 1276) were adapted as reference methods to compare the sensitivities of the bacterial strains recommended in the standards with those of particular concern in the dairy industry. The results of disinfectants achieved in suspension tests were also compared to results using bacteria attached to surfaces. *Bacillus* spores are of great concern to the dairies, spores were therefore included to study whether the disinfectants were able to kill spores in suspensions.

1.2 SUMMARY OF SOILING AND CLEANING PARAMETERS

A list of soiling parameters used in the experiments carried out in the project is given in Table 1. The experimens were performed in pilot scale on test rigs. The work was carried out in cooperation with representatives from VTT, Matforsk and SIK (Figure 1). Variables used in disinfectant testing performed at Matforsk are also summarized in Tables 1 and 2.

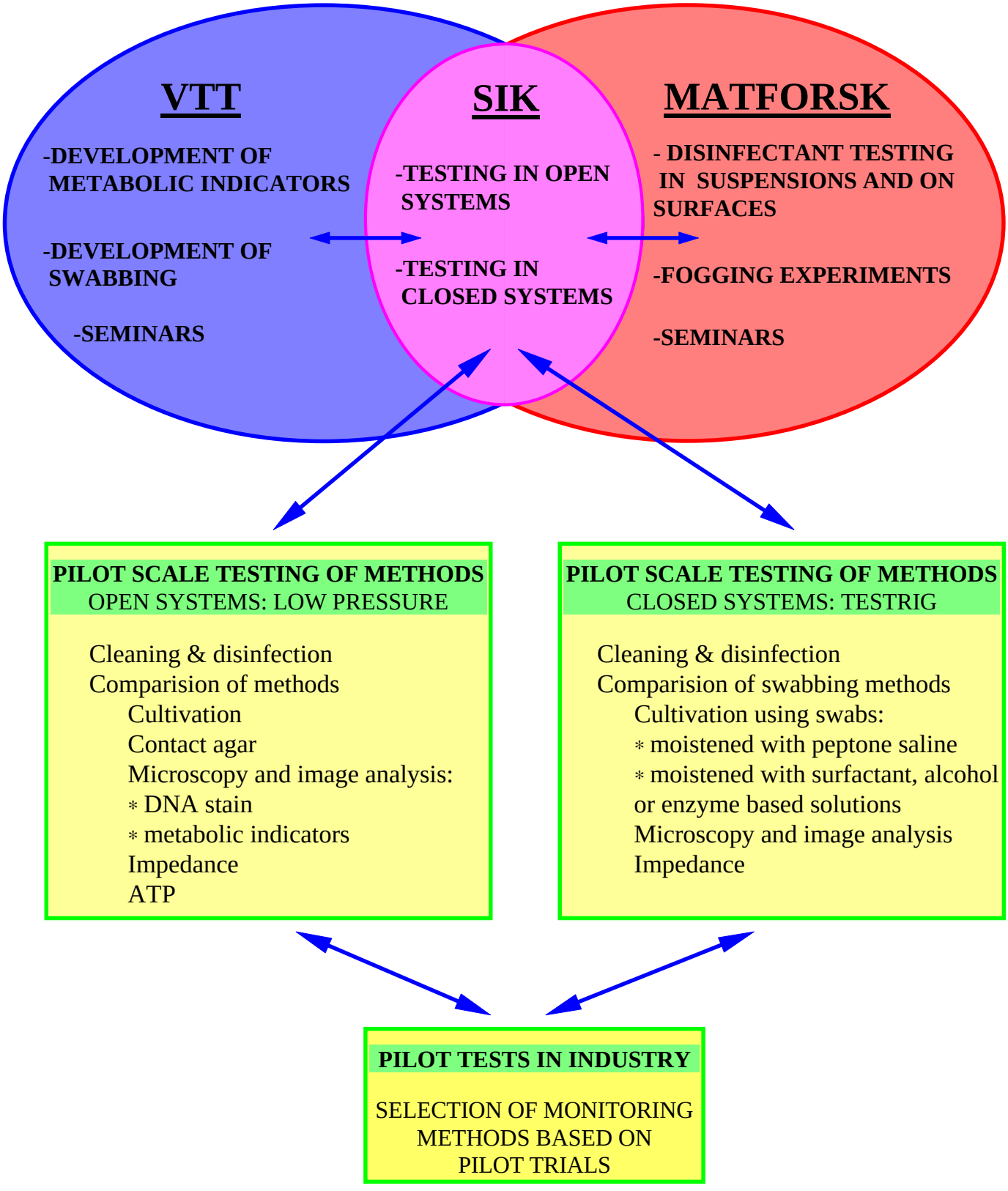

Figure 1. Cooperation in the NordFood project P93156 "Sanitation in dairies" in developing test methods for hygiene monitoring on equipment surfaces

Table 1. Priorities of soiling parameters and experimentals in the NordFood project P93156 "Sanitation of dairies"

Variables	Finland Valio & VTT	Norway Norske Meierier & Matforsk	Sweden SIK
Microbes	1) *Bacillus cereus* NCTC 2599 (SIK) 2) *Pseudomonas fragi* VTT E-86249 3) *Listeria monocytogenes* (Valio) 4) *Enterococcus hirae* VTT E-60046 5) *Salmonella infantis* VTT E-96733 ⇒ *Bacillus thuringensis* used in stead of no. 1 ⇒ pure and mixed cultures of strains no. 11 & 2 (closed systems) and no. 2 & 3 (open systems)	6) *Pseudomonas aeruginosa* (CEN) 7) *Staphylococcus aureus* (CEN) 8) *Bacillus cereus* (spore, Dutch 5-5-5) 9) *Enterococcus faecium* (CEN) 10) *Eschericia coli* (CEN) 11) *B. thuringensis* ⇒ strains no. 1 - 11 in suspension test ⇒ *P. fragi* used in biofilm tests	1) *B. cereus* (spores) 11) *B. thuringensis* (spores) 12) *B. subtilis* (spores) ⇒ strains no. 1 and 11are used in spore tests in closed systems performed at SIK. Strain no. 11 will be used in test runs performed at VTT
Soil	1) Sour milk containing fat (piimä) 2) NORDFOOD soil (1 % whole milk powder, 1 % whey powder & 0.5 % modified starch (E1422), 20 ml whipping cream, 80 ml water ⇒ NORDFOOD soil is a more appropriate soil	3)Suspension tests performed using skim milk powder	1) Sour milk containing fat 2) NORDFOOD soil 4) No soil except spores
Treatment of soil on surfaces	1) 75 °C-hot surfaces are dipped in cold product for 5 min and dried in 75 °C (repeated twice)	Same as mentioned by VTT & Valio	Same as mentioned by VTT & Valio
Surface materials	1) Stainless steel AISI 304, 2B (R_a 1.6) 2) EPDM rubber	Same as mentioned by VTT & Valio	Same as mentioned by VTT & Valio
Laboratory tests for cleaning agents & disinfectants	1) Surface test e.g. tests by Bloomfield *et al.* 2) Biofilm growth (young & old) on surfaces ⇒ <u>EHEDG test method</u>	3) Suspension test (CEN PrEN 1040 & CEN PrEN 1276) 4) Field tests	5) Surface test with spores

Table 2. Priorities of cleaning parameters and monitoring techniques used in the NordFood project P93156 "Sanitation of dairies"

Variables	Finland Valio & VTT	Norway Norske Meierier & Matforsk	Sweden SIK
Cleaning agents and methods in pen systems	Foam cleaning: 1) SU727 Trippel (alkaline & chlorine) 2) P3-topax 12 (mild alkaline) (3) SU628 Powergel (alkaline & tensides))	See chemicals mentioned by Valio & VTT	See chemicals mentioned by Valio & VTT
Cleaning agents and methods in closed systems	CIP treatment: flow rate 1.5 m/s 1) 1% sodium hydroxide (= NaOH) (70 °C, 7 min or 20 min) with or without 0.8% nitric acid 2) 1% NaOH with 1.2% P3-STABICIP EA 3) 1% NaOH with 0.2% SU560 4) 1.3% MIP SP (KOH based with chelators)	See chemicals mentioned by Valio & VTT	See chemicals mentioned by Valio & VTT
Disinfectants	1) P3-Oxonia aktiv 2) Virkon ⇒ disinfectant testing performed at VTT's test rigs in co-operation with Matforsk and SIK	3) P3-triquart (quat based) 4) IPA 300 (isopropanole & quats) 5) Hypokloran SP (alkaline & chlorine) 6) P3-Topax 99 (alcyl amine acetate) ⇒ suspension tests: agents no. 1 - 6	7) steam sterilization
Detection methods	1-3) Cultivation (plating techniques, contact plates, agar moulding) 4-5) Image analysis (epifluorescence microscopy) using acridine orange & metabolic indicators 6-7) Impedance measurement & ATP	8) Cultivation for suspension test see also methods no. 1 - 7	Methods no. 1 - 7 in experiments at the test rigs
Pilot plant studies	Based on laboratory experiments pilot trials were performed using both open & closed test rigs	Co-operation on the pilot plants at VTT	Co-operation on the pilot plants at VTT

1.3 CONTACT ADDRESSES OF PARTICIPANTS IN THE PROJECT

The contact addresses of the members in the research group and the industrial partners are given in Table 3.

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1.4 PURPOSE OF THE PRESENT STUDY

The purpose of the project was to harmonize the procedures used for testing of sanitation agents in Nordic dairies. The microbial methods used for the detection of biofilm and bacterial cells left on surfaces after cleaning and disinfection were conventional cultivation, image analysis after stainig with acridine orange or metabolic indicators (CTC-DAPI and LIVE/DEAD stains), impedance, mouling with TTC-agar and ATP measurement. Furthermore, the efficiency of cleaning agents and disinfectants used in dairies, were tested under controlled circumstances to enable comparison of the agents. The specific topics of the project were:

- testing of disinfectants in suspensions and on surfaces in laboratory scale,
- evaluation of monitoring methods for measuring cleaning efficiency of surfaces in open and closed systems,
- use of metabolic indicators in monitoring viability of microbial cells,
- use of detergent solutions for release of microbial cells from surfaces using both swabbing and spraying and
- testing of disinfection in industrial scale.

2 MATERIALS AND METHODS

2.1 DISINFECTANT TESTING

2.1.1 Suspension tests

Principle

In the European Standard Draft prEn 1040 a test suspension of bacteria is added to the disinfectant sample tested. The mixture is maintained at 20 °C ± 1 °C. After a specified contact time of e.g. 1, 5, 15, 30, 45 and 60 min ± 10 s the bactericidal action in the sample is immediately suppressed by neutralisation or by membrane filtration. The number of bacteria surviving in each sample is determined and the reduction in viable counts calculated.

In the Standard Draft prEN 1276 the requirements are stated that the bacterial suspension should be prepared in a solution of interfering substances. The bacterial suspension is added to the disinfectant under test diluted in standard hard water (WSH). In this study 1 and 10% (v/v) reconstituted skimmed milk and hard water with the hardness 300 mg($CaCO_3$) kg^{-1} water were used as interfering substances.

The test organisms in the draft standard prEN 1276:

Pseudomonas aeruginosa	ATCC15442
Escherichia coli	ATCC 10536
Staphylococcus aureus	ATCC 6538
Enterococcus faecium	ATCC 10541

Strains of concern in the dairy industry:

Enterococcus hirae	VTT E-60046
Pseudomonas fragi	VTT E-84200
Salmonella infantis	VTT E-96733
Listeria monocytogenes	Valio

Spores of concern in industrial environment:

Bacillus cereus	NCTC 2599
Bacillus thuringiensis	ATCC 10792

Preparation of the bacterial suspensions and disinfectant solutions

Streak-plates of each bacterial culture were incubated for 18-24 h at 37° C, except *P. fragi*, which was incubated at 20° C. After incubation, sterile glass beads and 3 ml 0.1% tryptone 0.85% sodium chloride solution (saline) were added to the bacterial sample. The petri dishes were gently rotated to loosen the bacteria from the agar. The resulting bacterial suspension was transferred to a sterile conical flask containing 20 ml tryptone saline. In prEn 1276 the optical density (OD) was measured using a spectrophotometer to adjust the bacteria in the suspensions to 1×10^8 - 3×10^8 CFU ml^{-1}. The disinfectant solutions were prepared in hard water at three different concentrations to include the active and non active range of the agents tested. In prEN 1040 the disinfectant solutions were prepared in distilled water. The disinfectants were: hypochlorite-based Hypokloran SP (Henkel-Ecolab), mild alkaline P3-Topax 99 (Henkel-Ecolab) containing alcylamineacetate, P3-Oxonia aktiv (Henkel-Ecolab) containing hydrogene peroxide and peracetic acid, quaternary ammonium agent P3-Triquart (Henkel-Ecolab), Virkon S (Antec International Ltd.) containing potassium persulphate and sulphamine acid and alcohol-based IPA 300 (Farmos Teknokemia) containing quaternary ammonium compound.

Procedure

One millilitre of reconstituted skimmed milk solution and 1.0 ml of bacterial test solution were pipetted into a sterile 100 ml conical flask, mixed and placed in a shaking water bath at 20° C $\pm$ 1° C for 2 min $\pm$ 10 s. Eight millilitres of the disinfectant were then added and shaked. Contact time was 5 min $\pm$ 10 s. The remaining procedure was as described above.

Interpretation of results

The results are given as a mean of at least two trials using a different set of sequential subcultures. In testing a disinfectant in accordance to prEN 1040, it is expected that the disinfectant demonstrates at least a reduction of 10^5 in viable count for both test organisms used after a contact time of max 60 min. By using the method in accordance with prEN 1276 a reduction is expected in the number of cells for the four reference stains from between $1 - 3 \times 10^7$ to no more than 3×10^2 CFU ml^{-1} within 5 min ($\geq 5 \log_{10}$ reduction).

2.1.2 Surface test

Principle

The suspension of the test bacterium was allowed to dry on a test surface. The solution of the disinfectant was then applied to completely cover the dried film of bacteria. After the exposure of surfaces to disinfectants for a specified period at a specified temperature the samples were transferred into a validated neutralizing solution, which immediately terminated the effect of the disinfectant. The number of surviving bacteria was determined. The method is presently under evaluation by the CEN/TC 216 test committee as a model standard tests.

Surface materials, test organism and disinfectants

The test surfaces (∅ 2 cm) of stainless cold-rolled steel (AISI 304, 2B) were cleaned using 20 ml of 5% v/v Decon 90 for 60 min before the tests. The coupons were then immediately rinsed with running distilled water for approximately 20 s to remove the surfactant and then sterilised in 95% n-propanol for 15 min. A test suspension of *P. fragi* VTT E-84200 was prepared using culture grown on Tryptone Soy agar at 20° C for 48 h. The microbial count in the suspension was adjusted to 2 - 6 x 10^9 CFU ml^{-1} through optical density measurements at 578 nm.

The disinfectants used in the testing were: P3-Oxonia Aktiv (peracetic acid containing peroxide), P3-Triquart (non - ionic tenside) and Virkon S (potassium persulphate chelate). The two first mentioned agents were diluted to highest and lowest recommended ready-to-use concentrations with distilled water or water of standard hardness (WSH) and Virkon S to the concentration given by the producer.

Procedure

The disinfectants, water, test bacterium and neutralizing solution (D/E neutralizing broth, Difco) were equilibrated in a water bath to 20° C ± 1° C. The bacteria were applied by pipetting 0.05 ml of the suspension containing 2 - 6 x 10^9 CFU ml^{-1} onto a test coupon, covering the entire surface with a thin film. The film was allowed to dry on the surfaces for 1 h at 20° C. The bacteria were exposed to the disinfectant by carefully pipetting 0.1 ml of the test solution onto the coupons and after a contact time of 5 min the surfaces were placed in 10 ml neutralizing solution containing 8 g glass beads. The surfaces were held at 20°C ± 1°C for 10 min ± 10 s under continuous shaking before plating a serial dilutions of the test mixture onto Tryptone Soy agar (incubation at 20°C ± 1°C, 48 - 72 h). Controls were performed using 0.1 ml distilled water or WSH instead of 0.1 ml test solution.

The microbiocidal effect of the test solution was calculated by subtracting the value of the coupon exposured to the disinfectant from the value of the control coupon in which the disinfectant had been substituted with distilled water or WSH.

2.2 CLEANING OF SURFACES IN OPEN AND CLOSED SYSTEMS

2.2.1 Open systems

Surface materials, test organisms and biofilm formation

The test surfaces used in the experiments were unsoiled or soiled cold-rolled stainless steel (AISI 304 2B) 25 x 75 mm^2 or 12 x 55 mm^2 coupons. The soil was NORDFOOD soil, which contained 10 g whey powder, 5 g modified starch (E1422), 10 g whole milk powder and 200 ml whipping cream (35% fat) in 800 ml sterile distilled water. The soiling of the test samples was carried out twice immersing prewarmed (75 °C) surfaces in the soil for 5 min. The soil was poured off and the surfaces dried at 75°C for 2 h.

The bacteria tested were *P. fragi, L. monocytogenes, B. thuringiensis* and a mixture of *P. fragi* and *L. monocytogenes*. Spore suspensions of *B. thuringiensis* and *B. cereus* were also tested. The bacteria were cultivated on Plate count agar (BBL, US). Bacterial suspensions were grown as described by Wirtanen and Mattila-Sandholm (1993, 1994). The different inocula contained approximately 10^7 - 10^8 CFU ml^{-1}. The spore suspensions were prepared as described by Husmark (1993) and contained approximately 10^7 spores ml^{-1}. The biofilm was allowed to develop on both unsoiled and soiled surfaces for 4 days in autoclaved slime-broth as described by Wirtanen and Mattila-Sandholm (1993, 1994). The slime broth contained 2.4 g Lab-Lemco powder (Unipath Ltd., UK), 8,0 g Nutrient broth (Unipath Ltd.), 50 g sucrose (BHD Laboratory Supplies, UK), 10 g glucose (BHD Laboratory Supplies) and 10 g fructose (Merck, Germany) per liter distilled water. Test surfaces were immersed in 200 ml of the slime broth inoculated with 2 ml of bacterial suspension. Incubations were terminated after four days by rinsing the test surfaces twice in sterile distilled water at room temperature before further treatment. Spore suspensions were sprayed directly onto the test surfaces and allowed to dry at room temperature for 2 h.

Cleaning procedures

The stainless steel surfaces with biofilm were cleaned using four different treatments with: 1) an effective foam cleaning agent, 2) an ineffective foam cleaning agent without disinfectant treatment and 3-4) an ineffective foam cleaning agent in combination with two different types of disinfectants. Positive controls without cleaning and negative controls incubated in sterile broth were included in the studies. Cleaning agents and disinfectants were: 3% Trippel SU 727 (Suomen Unilever Oy, Leverindus), 3% P3-Topax 12 (Henkel-Ecolab), 3% P3-Topax 12 in combination with disinfection using 1% P3-Oxonia Aktiv (Henkel-Ecolab) and 3% P3-Topax 12 in combination with disinfection using 1% Virkon S (Antec International Ltd. Suffolk, England)) [results of Virkon S are not shown]. Foam was applied for 15 min at a distance of approx. 30 cm from the surface and rinsing was performed with 20°C water with low pressure for 10 s (Lagafors Fabriks AB, Sweden).

Trippel is a foam-cleaning agent consisting of anionic-active tensides, organic complex formers, sodium hydroxide and hypochlorite. The working pH of Trippel was approx. 12.5. Topax 12 is a milder agent with a working pH of approx. 9.5. It is recommended for cleaning of painted surfaces, walls etc. The effect of disinfection with Oxonia Aktiv and Virkon S was studied after treatment with Topax 12, which left 10^3 CFU cm^{-2} on the surface. These results were compared with the one-stage washing treatment using Trippel. The concentration of the disinfectants was 1% (v/v) solution of Oxonia Aktiv or 1% (w/v) solution of Virkon S. The disinfection time was 5 min and disinfection was carried out at 20°C before a final low pressure rinse for 10 s with 20°C water at a flow of 27 l min^{-1}.

Microbial determinations

The microbial determinations were conventional cultivation and image analysis linked to epifluorescence microscopy of preparations stained with both acridine orange and metabolic stains, automated impedance measurements using the BacTrac 4100 equipment (Sy-Lab, Austria), moulding with TTC-agar and ATP measurements using the Surface Monitoring Kit 1243-114 (Bio-Orbit Oy, Turku, Finland). The metabolic stains were 5-cyano-2,3-ditolyl tetrazolium chloride [CTC] and 4', 6-diamidino-2-phenylindole [DAPI] as well as LIVE/DEAD *Bac*Light Viability Kit L-7007) [results not shown]. Three replicate samples of both unsoiled and soiled surfaces were prepared for each microbe, each treatment and each determination. The spores were determined using only moulding with TTC agar.

2.2.2 Closed systems

Surface materials, test organisms and biofilm formation

The test surfaces were treated as described in chapter 2.2.1. The bacteria tested were *P. fragi* and *B. thuringiensis*. The bacteria were cultivated on Nutrient agar (Difco, US). Bacterial suspensions were used for biofilm formation as described by Wirtanen and Mattila-Sandholm (1993, 1994). The different inocula contained approximately 10^7 - 10^8 CFU ml^{-1}. The spore suspensions were prepared as described by Husmark (1993) and contained approximately 10^7 spores ml^{-1}. The biofilm formation was carried out as described in chapter 2.2.1. Spore suspensions were sprayed directly onto the test surfaces and allowed to dry at room temperature for approx. 2 h.

Cleaning procedures

The stainless steel surfaces with biofilm were cleaned using four different single-phase treatments. Single-phase cleaning agents for CIP-treatment, are nowadays being more commonly used because the processing industry wants to save time. Positive controls without cleaning and negative controls (incubated in sterile broth) were included in the studies. Cleaning agents were: 1% sodium hydroxide (NaOH), 1% NaOH with 1.2% P3-

StabiCIP EA (Henkel-Ecolab) added, 1% NaOH with 0.2% SU560 (Lilleborg) added and 1.3% Mip SP (Henkel-Ecolab).

The CIP-procedure was carried out in the test rig (Tetra Pak Oy, Finland) at VTT. The diameter of the pipes is 51 mm (test section 63 mm), and the heating of the liquids is performed with steam. The volume of the pipes and the test section is 30 l. The test surfaces were placed in a rack in the vertical part of the system. The CIP procedures were carried out as follows: rinsing with cold water 2 min, heating of the water for the alkaline treatment to 75 ºC, alkaline treatment for 7 min and a final rinse for 2 min with cold water. The flow rate used in these trials was 1.5 m s^{-1}.

The microbial determinations performed were: four different swabbing detergents for cultivation, image analysis linked to epifluorescence microscopy after staining with acridine orange, automated impedance measurements using the BacTrac 4100 (Sy-Lab, Austria) equipment and moulding with TTC agar. Three replicate samples of both unsoiled and soiled surfaces were prepared for each microbe, each treatment and each determination.

2.3 TESTING OF METABOLIC INDICATORS

2.3.1 Bacteria in suspensions and on surfaces

All the tests were performed with three types of samples: biofilm grown on stainless steel coupons, bacterial cells grown in biofilms and swabbed before further treatments and bacterial cells in suspension. The metal surface used in these tests was stainless steel (AISI 304, 2B). The bacteria tested were *P. fragi* and *L. monocytogenes,* which were cultivated on Plate count agar (Difco, USA).

Autoclaved slime broth was used as a test matrix to obtain the nutrients needed for biofilm formation. 50 ml of slime broth in an Erlenmeyer flask was inoculated with 0.5 ml bacterial suspension. The suspension was grown for 18 h at 25°C, during which time the Erlenmeyer flask was shaken 60 r min^{-1}. The biofilm was allowed to develop on the coupons for 2 d at 25°C (60 r min^{-1}) in inoculated slime broth. Test coupons were removed after 2 d and rinsed twice with sterile, distilled water for 15 s at room temperature before the measurements using metabolic indicators. Cultivation and ATP measurement were used as reference methods in this evaluation.

2.3.2 Experimental and staining procedures

LIVE/DEAD Bac*Light viability kit*

Staining with LIVE/DEAD *Bac*Light Viability Kit (Molecular Probes, Inc., USA) was performed using a mixture of Reagent A and Reagent B (ratio 1:1) for suspension and swabbed biofilm bacteria. This staining solution for suspension was diluted in a ratio of 1:2 with sterile-filtered double distilled water.

Bacterial suspension or swabbed biofilm bacteria (1 ml) was added to an Eppendorf tube, and stained with 6 µl LIVE/DEAD stain for 15 min in the dark at room temperature. After incubation, 5 µl of the stained suspension was trapped between a glass slide and a 24 mm^2 coverslip for immediate microscopic observation using suitable filter combination.

The entrapment technique was useful for analysing the bacterial suspension. Some experiments were performed with swabbed biofilm bacteria using both sides of the coupon and reducing the volume. In practice this volume was not sufficient to proceed with staining, cultivation, and ATP measurement.

The LIVE/DEAD samples were observed under epifluorescence microscopy, using B-, U-, and V-filters of which the B-filter gave the best results. When using U- and V-filters the cells were blue-green and the color differences not as clear as when using the B-filter. Both living and dead bacteria fluoresced, making it possible to use image analysis for analysing samples. The total number of cells was counted with image analysis, using the black-and-white video camera available. The number of green-fluorescing living cells or red-fluorescing dead cells was counted manually depending on the type of cells, which was less.

Staining with CTC and DAPI

Bovill *et al.* (1994) found that the optimal CTC concentration varied 4-7 mM. The CTC concentrations of 4 mM for bacteria in suspension and swabbed biofilm bacteria and 5 mM for biofilm were chosen based on these findings. Schaule *et al.* (1993) and Yu and McFeters (1994) found that 2 h was the optimum incubation time for CTC. The content of one vial of the CTC stain was dissolved sterile-filtered double distilled water to a final concentration of 50 mM. The DAPI concentration used was 1 $\mu g\ ml^{-1}$ in sterile-filtered double distilled water.

Modified staining of biofilm with CTC and DAPI was performed according to Yu and McFeters (1994). The stainless steel biofilm coupon was placed in a Petri dish, and staining with 2 ml of 5 mM CTC was performed for 2 h at 37 ± 1°C without shaking. After incubation, the stain was removed and replaced with 2 ml of 5% formaldehyde (Riedel-de Haën) to fix the CTC stain. The samples were incubated in formalin for 5 min at room temperature. Thereafter they were stained with 2 ml of 1 $\mu g\ ml^{-1}$ DAPI. The sample was incubated for 3 min at room temperature, after which the stain was removed. The coupons were rinsed with sterile distilled water, air-dried, and stored at 4 ± 1°C for microscopic observation.

CTC staining of suspension and swabbed biofilm bacteria was performed as follows: 1 ml of suspension or swabbed biofilm was added to a test tube, followed by an addition of 0.1 ml of 50 mM CTC solution. The sample was incubated at 30 ± 1°C for 2 h. After CTC staining, the samples were separated through centrifugation (1602 x *g*, 15 min) and 1 ml of

1 μg ml^{-1} DAPI was added to the Eppendorf tube. The sample was incubated for 20 min at room temperature. After incubation, 5 μl of the stained suspension was trapped between a glass slide and a 24 mm^2 coverslip for immediate microscopic observation. The swabbed biofilm bacteria samples were filtered and stored at 4 ± 1°C before examination.

The CTC-DAPI samples were observed under epifluorescence microscopy using B-, U-, and V-filters, of which the B-filter gave the best results, showing the presence of green-fluorescing cells with DAPI. The cells were blue-green with the U- and V-filters and the fluorescence brighter than with the B-filter. Since CTC was the metabolic indicator and was more visible with the B-filter, the B-filter was used (Stewart *et al.* 1994; Yu and McFeters, 1994; Huang *et al.,* 1995).

2.4 DETECTION METHODS

2.4.1 Conventional cultivation

Bacteria were scraped from the test surfaces (2.5 x 7.5 cm^2) with a cotton-tipped swab, which was transferred into a test tube containing 5 ml peptone saline (LabM, UK) or, for disinfected surfaces in inactivation solution, which consisted of 0.6 g lecithin (Merck, Germany), 6.0 ml Tween 80 (Fluka Chemical, Switzerland), 0.8 g sodium thiosulfate ($Na_2S_2O_3 \cdot 5\,H_2O$; Merck), 0.1 g L-histidine hydrochloride ($C_6H_{10}ClN_3O_2 \cdot HCl \cdot H_2O$; Merck), 1.0 ml phosphate buffer 0.25 mol l^{-1} (3.4 g potassium hydrogen phosphate (KH_2PO_4; Merck) in 100 ml distilled water), 0.72 g bovine albumin (Sigma, USA) in 100 ml distilled water. The test tube containing the swab was vortexed to release cells into the medium. The samples were diluted in logarithmic series, plated onto Plate Count agar (Difco, US) and incubated for 48 h at either 30°C (*P. fragi*, *B.thuringiensis* and the mixture of *P. fragi* and *L. monocytogenes*) or at 37 °C (*L. monocytogenes*).

2.4.2 Image analysis

The staining of the surfaces and the image analysis were performed as described by Wirtanen and Mattila-Sandholm (1993, 1994). 0.01% solution of acridine orange (Difco, UK) was poured onto the test surfaces and allowed to act for 2 min at room temperature, after which the surfaces were rinsed with sterile water, air-dried for 24 h and stored in the dark at +4°C. The image analysis was carried out with the CUE-2 planomorphometry program (Galai Production Ltd., Israel) using a microcomputer system connected to an Olympus BHT epifluorescence microscope (Japan) with a suitable filter combination. The microcomputer consisted of a camera (Hamatsu Photonics K.K., Japan), a SONY trinitron superfine pitch image monitor with the program ADI DM-3114 and a printer (Panasonic, Japan). The images obtained were analysed as grey scale interpretations on the screen. The areas covered by biofilm and/or cells were converted into percentages of the total area.

50 microscopic fields per coupon were examined to obtain a mean value of the coverage. The metabolic indicator CTC-DAPI was also used in the low pressure experiments. The parameters used in these experiments are given in chapter 2.3.2. In these experiments 120 microscopic fields/coupon were examined.

2.4.3 Cultivation combined with swabbing using novel detergents

The remaining biofilms were also detected by swabbing the surfaces with swabs moistened in detergent solutions or with dry swabs with detergents sprayed on sampling surface. The main components in the detergent solutions tested were surfactants, alcohols together with abrasive chemicals and enzymes together with abrasive chemicals. The swabs were placed in inactivation solution, which was used to neutralize the chemicals interfering with the cultivation. The Hygicult® contact slides were inoculated with samples from equipment parts moistened with detergent solutions sprayed on the surfaces. Sampling was performed using Hygicult® TPC (Total Bacteria Count) and Hygicult® Y&F (Yeasts & Fungi) slides from Orion Diagnostica Ltd. (Finland).

2.4.4 Contact plates and agar moulding using TTC-agar

An amount of 800 µl tetrazolium chloride (TTC) was added to 200 ml tryptone glucose extract agar (Difco). The agar was poured onto the test coupons and incubated overnight. The agar was then removed from the test surface, placed with the contact-surface uppermost in a sterile petri dish and incubated further at either 30°C (*P. fragi, B. thuringiensis*, spores *of B. thuringiensis* and the mixture of *P. fragi* and *L. monocytogenes*) or 37°C *(L. monocytogenes* and *B. cereus* spores).

2.4.5 Impedance

The change in impedance (resistance) in the growth medium caused by microbial growth was measured automatically using a BacTrac 4100 instrument (Sy-Lab, Austria). The instrument consists of autoclavable glass measuring vessels with 2 pairs of electrodes, which are connected to a microprocessor. The growth of microorganisms changes the concentration of ions in the growth medium and in the layers surrounding the electrodes. Changes in conductance, the M-value (relative Medium impedance) are brought about by bacterial metabolism, weakly charged substrates in the growth medium are transformed into highly charged end products. The growth of some microorganisms does not result in large changes in conductance (M-value), the growth can also be detected through measuring changes in capacitance (E-value = relative Electrode impedance). Monitoring of the E-value should be used in case the growth medium contains high concentrations of salts, which makes the M-value inaccurate. The test surfaces (12 x 55 mm^2) were placed in the measuring vessels each of which contained 9 ml growth medium. The measuring vessels were incubated at 30°C for 48 h and comparisons were made of the time taken by each sample to reach the same E-value.

2.4.6 ATP determination

The amount of ATP in the biofilm on the coupons was measured using the Surface Monitoring Kit 1243-114 (Bio-Orbit Oy, Turku, Finland). A sterile swab was moistened in 0.85% sodium chloride, the surfaces was swabbed and the swab was vortexed in 500 µl of ATP Releasing Buffer for 2 min in a measuring cuvette after which 500 µl of ATP Monitoring reagent was added. The cuvette was vortexed and measured in the Bio-Orbit 1253 Luminometry system.

2.4.7 Summarization of main methods

Table 4. Methods used in assessing the cleaning efficiency of process surfaces.

Detection method	**Application**
Conventional cultivation	assesses living bacteria
Image analysis	assesses biofilm components on the surface (including organic soil, dead and living cells, and slime)
Impedance	assesses the activity of viable and injured bacteria which can recover after reactivation in broth

2.5 TESTING OF DISINFECTION IN INDUSTRIAL SCALE

The first part of the studies was carried out at Rogalands meieriet, Voll, in a packaging room for rindless cheese during a period of eight weeks in the autumn 1995. Removal of mould from packaged cheese and repackaging was also carried out in these facilities. The ventilation system was new meeting the standards of the dairy industry. Cleaning procedures including daily foaming with P3-TP66 (Henkel-Ecolab) on floor and equipment and rinsing with water was carried out. Disinfection with 2 - 3% P3-TP99, a mild alkaline disinfectant with alcylamine acetate as active agent, using a fogging equipment P3-Des-O-Mat (Henkel-Ecolab) with two clusterheads with 8 nozzles each, was performed. The fogging program was as follows:spreading of disinfectant for 20 min, exposure for 20 min and water rinse for 20 min. The intention was to investigate the influence of the frequency on the effect.

The second part of these studies was carried out at TINE Midt-Norge avd. Ørland in cheese making and packaging facilities for rindless cheese during spring 1996. The fogging equipment used was P3-Des-O-Mat and the disinfectant was P3-TP99. The program and concentration were identical with the parameters used in the experiments at Rogalands meieriet. The microbial levels were investigated for four weeks without fogging followed by a periode with fogging. This experiment was followed by a two weeks test after three months of fogging to find out, if there were any changes in microbial levels. A test with coupons inoculated with bacteria was also carried out in connection with the last experiment.

3 RESULTS AND DISCUSSION

3.1 DISINFECTANT TESTING

3.1.1 Suspension tests

Results indicated a higher than 5 $\log_{10}$ reduction in viable count after 5 min of exposure to 80% of the lowest recommended concentration of the six disinfectants in distilled water and without organic load added. The disinfectants containing potassium persulphate and isopropanol were only recommended for use at one concentration, but are included in all the tables for comparison. Thus the prEN 1040 suspension test did not differentiate between the six disinfectants, which was to be expected since the prEn 1040 procedure is designed to test wheather formulations are effective disinfectants. All commercial disinfectants are supposed to pass the requirement of the test at the lowest recommended concentration. Practical situations with e.g. porous surfaces, disinfectant liquid contining organic soil, hard water and dead ends with bacterial biofilm attached challenge the effect of disinfectants. More stringent tests than the one proposed in Draft prEN 1040 are clearly needed to simulate the conditions of practical application.

The draft prEN 1276 standard is designed to address some of the anticipated difficulties of practical applications by using disinfectants diluted in WSH and the inclusion of organic soil in the test. In the presence of 1% (v/v) skim milk, the highest recommended concentration of each of the six disinfectants resulted in ≥ 5 $\log_{10}$ reduction in numbers of the four test organisms. However, at their lowest recommended concentrations and in the presence of 1% (v/v) skim milk, each disinfectant gave < 5 $\log_{10}$ reduction in numbers of at least one of the test organisms (Table 5).

Table 5. Comparison (log_{10} reduction) of efficiency of disinfectants using the lowest recommended concentration in water of standard hardness on standard strains proposed in prEN 1276.

Disinfectants / Strains	Hypo-kloran SP	P3-Topax 99	P3-Oxonia aktiv	P3-Triquart[a]	Virkon S	IPA 300[b]
Staphylococcus aureus	< 1	> 5	> 5	> 5	> 5	> 5
Pseudomonas aeruginosa	< 1	4.4	3.5	< 1	> 5	> 5
Enterococcus faecium	> 5	> 5	3.6	> 5	> 5	> 5
Escherichia coli	3.8	> 5	> 5	> 5	> 5	> 5

a. Quaternary ammonium compound. Skim milk (1% v/v) was included during exposure of 10^7 cells ml^{-1}. When tested in distilled water without skim milk protein, all disinfectants gave ≥ 5 $\log_{10}$ kill of all strains.
b. Alcohol-based agent containing quaternary ammonium

E. faecium and *E. coli* were the most sensitive of the four standard strains, showing a 5 $\log_{10}$ reduction in the presence of five of the six disinfectants. *S. aureus* showed marked resistance to the chlorine compound under these test conditions, and *P. aeruginosa* to both the chlorine compound and the quaternary ammonium compound containing non ionic tenside formulation. Thus differentiating between the efficiency of some disinfectants is possible.

It is well known that quaternary ammonium compounds are affected by WSH and chlorine by skim milk, but disinfectants may contain components which counteract this effect. High levels of interfering compounds are relevant for places difficult to wash e.g. dead ends. Increasing the concentration of skim milk to 10% (v/v) reduced the efficiency of the majority of disinfectants (Table 6), but to variable degrees. All four standard strains appeared more resistant than the strain chosen by the dairy representatives, particularly *P. aeruginosa* and *S. aureus*.

Table 6. Comparison (log_{10} reduction) of efficiency of disinfectants using the lowest recommended concentrations in water of standard hardness and skim milk (10% v/v).

Disinfectant / Strain	Hypo-kloran SP	P3-Topax 99	P3-Oxonia aktiv	P3-Triquart[a]	Virkon S	IPA 300[b]
Staphylococcus aureus	0.7	1	3	3	3.6	> 5
Pseudomonas aeruginosa	0.4	0.5	3.4	0.1	> 5	> 5
Enterococcus faecium	> 5	0.5	3.5	3.5	> 5	> 5
Escherichia coli	0.4	0.7	> 5	0.7	> 5	> 5
Enterococcus hirae	> 5	< 1	4.3	4.1	> 5	> 5
Pseudomonas fragi	< 1	1.1	> 5	0.3	> 5	> 5
Salmonella infantis	< 1	< 1	> 5	< 1	> 5	> 5
Listeria monocytogenes	> 5	3.9	> 5	4.4	> 5	> 5

a. Quaternary ammonium compound. Skim milk (1% v/v) was included during exposure of 10^7 cells ml^{-1}. When tested in distilled water without skim milk protein, all disinfectants gave ≥ 5 $\log_{10}$ kill of all strains.
b. Alcohol-based agent containing quaternary ammonium

Using the highest recommended concentrations and 10% (v/v) skim milk, all test organisms except the two *Enterococcus* spp. and the *L. monocytogenes* showed some degree of resistance to one or more of the disinfectants (Table 7). If diluted to 80% of the lowest recommended concentration, only the isopropanol-containing disinfectant resulted in > 5 $\log_{10}$ reduction for all strains (Table 8).

Of the dairy strains chosen *S. infantis* was the most resistant (Tables 5 - 8). However, it was less resistant than either *P. aeruginosa* or *S. aureus*, which are recommended for use in the Draft European Standard. Therefore, it is probably not necessary to include separate test

strains when testing disinfectants for use in dairies. However, the significance of resistance in bacterial strains developed using disinfectants should also be evaluated.

Testing using prEN 1040 will not be expected to give unambiguous results which can aid in selecting a proper disinfectant for a dairy. Tests using prEN 1276, with WSH and skimmed milk could be a more stringent test, but the relevance of the results must be further evaluated in practical tests in the dairy. Suspension tests are applied to an inoculum of planktonic cells, while in the dairy industry bacteria are often attached to surfaces, making them more resistant to disinfection. Moreover, attachment with following growth on surfaces causes many bacteria to produce extensive amounts of extracellular polymers which envelope the attached cells. The activity of disinfectants could be substantially reduced by the presence of these protective exopolymers. In selecting disinfectants, aspects such as environmental effect, corrosiveness and possible resistence development must also be considered. Disinfection is generally understood to mean the destruction of vegetative microorganisms, but not necessarily endospores. In the Dutch 5-5-5 Suspension Test Method, the lowest concentration required to reduce the number of spores one $\log_{10}$ using 5 min contact time is determined for a disinfectant claimed to be sporicidal. According to this requirement, none of the disinfectants were sporicidal even when used at the highest recommended concentration.

Table 7. Comparison (log_{10} reduction) of efficiency of disinfectants using the highest recommended concentrations in water of standard hardness and skim milk (10% v/v).

Disinfectant / Strain	Hypo-kloran SP	P3-Topax 99	P3-Oxonia aktiv	P3-Triquart[a]	Virkon S	IPA 300[b]
Staphylococcus aureus	2.3	> 5	> 5	> 5	3.6	> 5
Pseudomonas aeruginosa	3.4	> 5	> 5	3.8	> 5	> 5
Enterococcus faecium	> 5	> 5	> 5	> 5	> 5	> 5
Escherichia coli	1.6	> 5	> 5	> 5	> 5	> 5
Enterococcus hirae	> 5	> 5	> 5	> 5	> 5	> 5
Pseudomonas fragi	3.6	> 5	> 5	> 5	> 5	> 5
Salmonella infantis	< 1	> 5	> 5	1.7	> 5	> 5
Listeria monocytogenes	> 5	> 5	> 5	> 5	> 5	> 5

a. Quaternary ammonium compound. Skim milk (1% v/v) was included during exposure of 10^7 cells ml^{-1}. When tested in distilled water without skim milk protein, all disinfectants gave ≥ 5 $\log_{10}$ kill of all strains.
b. Alcohol-based agent containing quaternary ammonium

Table 8. Comparison (log_{10} reduction) of efficiency of disinfectants using 80% of the lowest recommended concentrations in water of standard hardness and skim milk (10% v/v).

Disinfectant / Strain	Hypo-chloran SP	P3-Topax 99	P3-Oxonia aktiv	P3-Triquart[a]	Virkon S	IPA 300[b]
Staphylococcus aureus	0	1.1	2.3	3.1	2.2	> 5
Pseudomonas aeruginosa	0.2	0	2.9	0.1	> 5	> 5
Enterococcus faecium	> 5	0.6	3.2	3.5	4.7	> 5
Escherichia coli	< 1	0.6	> 5	0.6	> 5	> 5
Enterococcus hirae	> 5	< 1	0.6	2.4	3.4	> 5
Pseudomonas fragi	< 1	1.1	3.5	0.2	> 5	> 5
Salmonella infantis	< 1	< 1	4.2	< 1	5	> 5
Listeria monocytogenes	> 5	> 5	4.2	2.6	4.8	> 5

a. Quaternary ammonium compound. Skim milk (1% v/v) was included during exposure of 10^7 cells ml^{-1}. When tested in distilled water without skim milk protein, all disinfectants gave ≥ 5 log_{10} kill of all strains.
b. Alcohol-based agent containing quaternary ammonium

3.1.2 Surface test

The results of the trials are presented in Table 9. As shown in the table, 0.2% peracetic acid containing peroxide gave > 5 log_{10} reduction of *P. fragi* using distilled water as diluent. All trials were conducted using WSH as a diluent. The lowest recommended user concentration (0.2% v/v) of peracetic acid containing peroxide gave an average reduction of 1.7 log_{10} whereas the highest user concentration (1% v/v) gave > 5 log_{10} reduction. Potassium persulfate chelate gave > 5 log_{10} reduction of *P. fragi* using the recommended ready-to-use concentration of 1%. The efficiency of potassium persulfate chelate was reduced if the disinfectant was left exposed to the light and/or air. Similar results were observed with the peracetic acid containing peroxide thus emphasising the importance of correct storage and use of the products. The lowest user concentrations of the non ionic tenside gave a reduction of < 1 log_{10} whereas the highest recommended concentrations gave < 5 log_{10}.

In contrast to the suspension test, where even in the presence of skim milk, *P. fragi* was reduced ≥ 5 log_{10}, this was not always the case in the surface test in the presence of only WSH. Thus the lowest recommended user concentration of the peracetic acid - based agent gave an average reduction of 1.7 log units and the highest user concentrations of the non - ionic tenside gave an average reducton of less than 1 log_{10} unit, which is in agreement with the findings of Bloomfield *et al.* (1993).

Table 9. Comparison of disinfectant efficiency in suspension and surface tests using log_{10} reduction. In the suspension test 10% skim milk was used as an organic load, no organic load was used in the surface test. The disinfectants were diluted in water of standard hardness in both tests.

Disinfectant / Test	P3-Oxonia Aktiv		P3-Triquart[a]		Virkon S
	Lowest recommended concentration	Highest recommended concentration	Lowest recommended concentration	Highest recommended concentration	Recommended concentration
Suspension	> 5	> 5	< 1	> 5	> 5
Surface	1.7	> 5	< 1	2.9	> 5

a. Quaternary ammonium compound.

According to the requirements of the CEN TC 216 test the difference between the test suspension and the test suspension with water added should not be greater than 2 log_{10}. The values obtained in these trials varied between 1.7 and 2.5. The difference between the neutralizing control and the the neutralizing test with disinfectant should not be greater than 0.3 log_{10}, which was true for most of the tests performed. The difference between the values of test suspension and neutralizing control should not be greater than 2 log_{10}, the values varied between 1.7 log_{10} and 4.0 log_{10} and exceeded 2 log_{10} in most of the tests. A possible explanation of this may be the exposure for 30 min to neutralizing agent.

3.2 CLEANING OF SURFACES IN OPEN AND CLOSED SYSTEMS

3.2.1 Open systems

Conventional cultivation

More bacteria were generally recovered from the soiled than from the unsoiled surfaces after cultivation using nutrient agar. Both *P. fragi* and *L. monocytogenes* attached well to the clean surfaces and even better to the soiled surfaces. Only a small number of *B. thuringiensis* were retrieved from the unsoiled surfaces, the number retrieved from the soiled surfaces was slightly higher. Thus *B. thuringiensis* either did not attach to the surfaces as well as the other test bacteria or was more difficult to remove by swabbing. The difficulty in obtaining more precisely reproducible biofilm is probably due to a number of factors which are difficult to control e.g. unevenness of the surfaces, position in the cultivation jar and circumstances during biofilm formation. The conditions found in nature are even more variable.

The cultivation performed on surfaces treated with Trippel and low pressure rinsing gave less than 10 CFU cm^{-2} of the test bacteria from both unsoiled and soiled surfaces. The

cultivation results showed that application of Topax 12 followed by low pressure rinsing reduced the number of colony forming bacteria on both unsoiled and soiled surfaces being $\log_{10}$ 1 - 3 CFU cm^{-2} after the cleaning treatment. The initial number of attached *B. thuringiensis* bacteria/spores was lower than the number of cells in the biofilm samples, which lead to a reduction of cells to less than 10 CFU cm^{-2} (= 1 $\log_{10}$ unit; results not shown) after the cleaning treatment.

The cultivation results also showed that the disinfection treatments after application of Topax 12 did not kill all the bacteria on the surfaces tested. According to these results, it appeared that Oxonia Aktiv was the most effective agent against *L. monocytogenes* and Virkon S against both spores and biofilm of *B. thuringiensis*. The effect of both disinfectants was small on biofilms of *P. fragi.*

Microscopy and image analysis after staining with acridine orange

Results from microscopy and image analysis after staining with acridine orange are shown in Figure 2. This method measured area percentage covered by biofilm including cells and is therefore useful in assessing the amount of biofilm left on the surface. There is good agreement between the results of the image analysis and conventional cultivation. Trippel SU 727 was more effective in removing biofilm in all cases with the exception of *B. thuringiensis* on the unsoiled surfaces. As would be expected the disinfection after cleaning with Topax 12 did not remove biofilm. The differences in the areas measured after the different disinfection treatments can therefore be regarded as parallel washing treatments. The variations in area coverage in parallel samples after washing is most probably a result of uneven distribution of biofilm on the surfaces.

Microscopy using image analysis after staining with *CTC and DAPI*

Results from the microscopy using image analysis after staining with the metabolic stain CTC in combination with DAPI stain are shown in Figure 3. According to the results the treatments were more efficient on soiled coupons. The CTC and DAPI staining showed that the number of viable bacteria remaining on the coupons was approximately 10^3 CFU cm^{-2} in nearly all cases after low pressure application of Topax 12, which was in agreement with the required number of bacteria on the surfaces prior to disinfection. The total number of bacteria on the surfaces (viable and dead cells) was somewhat higher. One interpretation of the results of the cultivation and the CTC-DAPI results may be that the swabbing procedure prior to cultivation may not be efficient enough to detach and recover the cells from the surface. Alternatively the effects of the disinfection and/or washing treatments could be to render the bacteria into a viable but nonculturable state with a potential for recovery or a combination of these factors. General comparison of the results after conventional cultivation and the CTC-DAPI staining suggested underestimations of the numbers of viable bacteria using conventional cultivation.

The results of the CTC-DAPI staining performed in the tests are shown in Figure 3. According to these results, Topax 12 in combination with Oxonia Aktiv was the most efficient combination for biofilms of *L. monocytogenes* and mixed culture of *P. fragi* and *L. monocytogenes* on unsoiled surfaces. On the other hand Topax 12 was the most efficient for biofilm of mixed culture of *P. fragi* and *L. monocytogenes* on presoiled surfaces and Trippel for *L. monocytogenes* biofilm on presoiled surfaces. The CTC-DAPI stainings were also performed with biofilms of *P. fragi* and *B. thuringiensis* (results not shown). The number of viable bacteria seemed to decline more after cleaning treatments of biofilms grown on soiled surfaces than on unsoiled ones. According to these staining results no overall preferable cleaning agent was found.

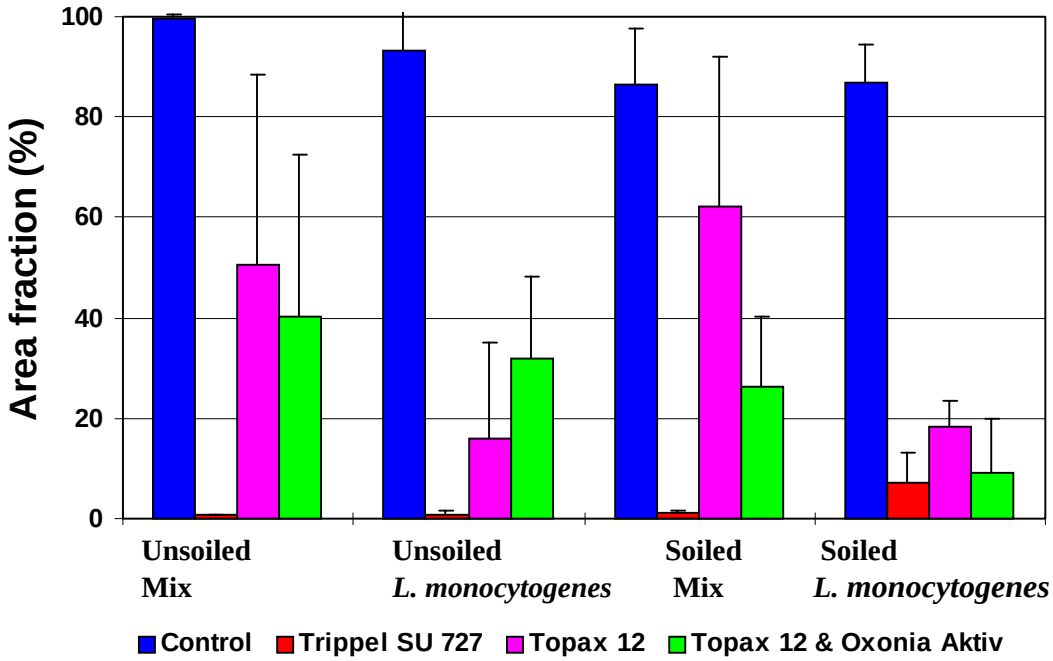

Figure 2. *The image analysis, after staining with acridine orange, of 4-d-old biofilms of* Listeria monocytogenes *and a mixed culture of* Pseudomonas fragi *and* L. monocytogenes *(mix) on unsoiled and soiled stainless steel surfaces (AISI 304, 2B) showed that the strong alkaline cleaner Trippel SU 727 was the most efficient agent in removing biofilms using low pressure cleaning.*

Moulding with TTC-agar

These results are difficult to interpret particularly in comparison with the results of the other methods used in this experiment. However they suggest that the *in situ* application of nutrient agar may facilitate recovery of cells that are damaged or altered in some way as a result of the treatments. The edges of the coupons gave a colour reaction indicating bacterial growth, which is not surprising as it is unlikely that the foam covered the edges sufficiently.

Impedance

Results from the impedance measurements are shown in Figure 4. The impedance results complemented the results of microscopy and cultivation. The recovery of *L. monocytogenes* and the mixed culture of *L. monocytogenes* and *P. fragi* took longer time after the treatments with Trippel SU 727 and Topax 12 in combination with disinfectant treatment, when compared to samples treated with only Topax 12. All of the cleaning procedures had some effect on *P. fragi* and resulted in longer recovery times than the control. This may be interpreted as a reduction in the number of bacteria after each treatment. The relatively high impedance values may also reflect the slower growth rate of *P. fragi* at 30°C in comparison with the growth rates of the other organisms tested. *B. thuringiesis* gave low detection times in the impedance measurements which are assumed to be due to a fast growth rate rather than the numbers of attached cells/spores (results not shown).

ATP measurement

This method was not sensitive enough for the purposes of the experiment carried out in this project. This method was therefore excluded in future experiments. If the results are to be presented in graphical form they should be within the limits 0.1 - 10 RLU when using the Bio-Orbit 1253 Luminometry system.

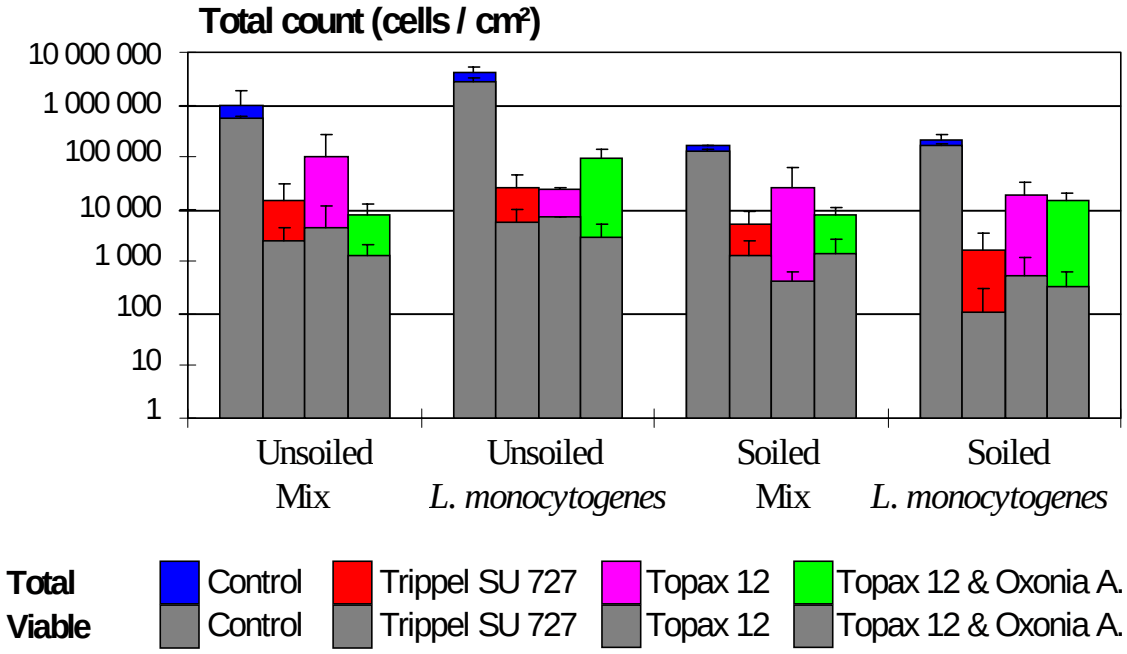

Figure 3. Image analysis after CTC-DAPI staining of Listeria monocytogenes *and a mixed culture of* Pseudomonas fragi *and* L. monocytogenes *(= mix) grown as biofilms on unsoiled and presoiled stainless steel surfaces (AISI 304, 2B) showed that most of the bacteria on the surfaces were non-culturable after the cleaning.*

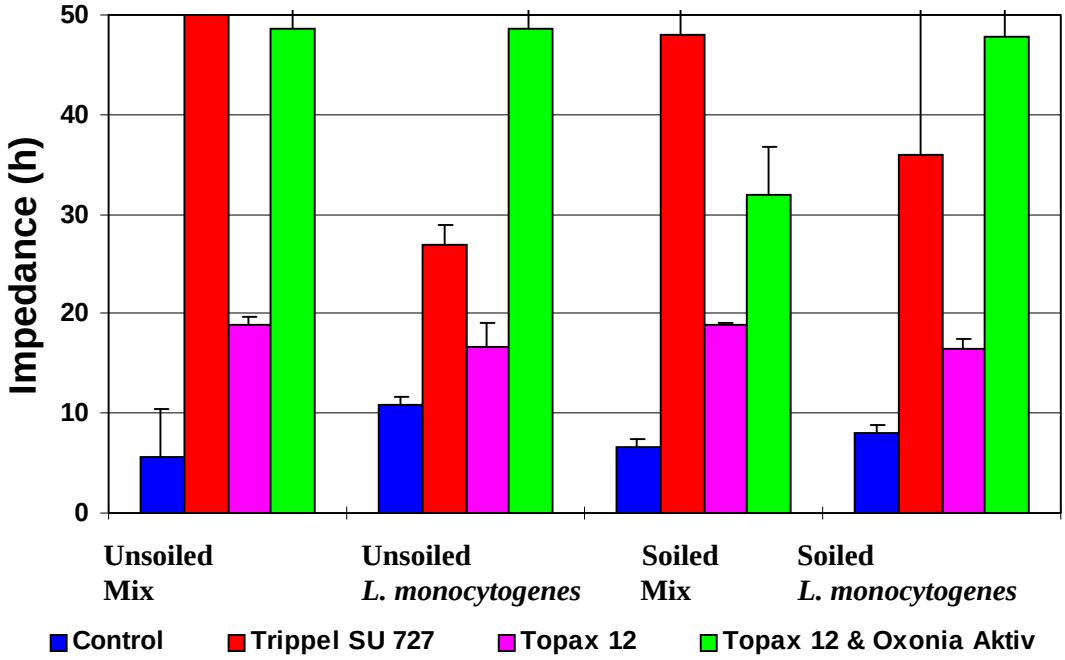

Figure 4. The impedance measurements of Listeria monocytogenes *and a mixed culture of* Pseudomonas fragi *and* L. monocytogenes *(= mix) grown as biofilms on unsoiled and soiled stainless steel surfaces showed that chlorine and disinfectant treatments prolonged the reactivation period of the treated cells in the biofilms.*

3.2.2 Closed systems

Conventional cultivation

The cultivation results given in Figure 5 showed that there were more cells of *P. fragi* on the reference surfaces than *B. thuringiensis* or spores of *B. thuringiensis*. These results showed that the treatments did not kill all the bacteria on surfaces tested. After the cleaning treatments there were less than 10 CFU cm^{-2} on both unsoiled and soiled surfaces. The results of the both reference samples showed that there was a clear difference between unswabbed and swabbed samples, but after the treatments the differences were not so clear.

Microscopy and image analysis after staining with acridine orange

The image analysis of surfaces stained with acridine orange measures the percentage of the area covered by biofilm including cells and is useful in assessing the amount of biofilm left on a surface. The results are given in Figure 6. The positive controls showed enormous variation between unswabbed and swabbed biofilm bacteria. The results after cleaning treatments and before swabbing of surfaces showed that biofilm was left on the surfaces. There was a considerable amount of residual organic material after the sodium hydroxide procedures. The differences on unsoiled surfaces were not so clear. For soiled surfaces the cleaning using MIP SP (a potassium hydroxide based cleaning agent) solution was the most effective.

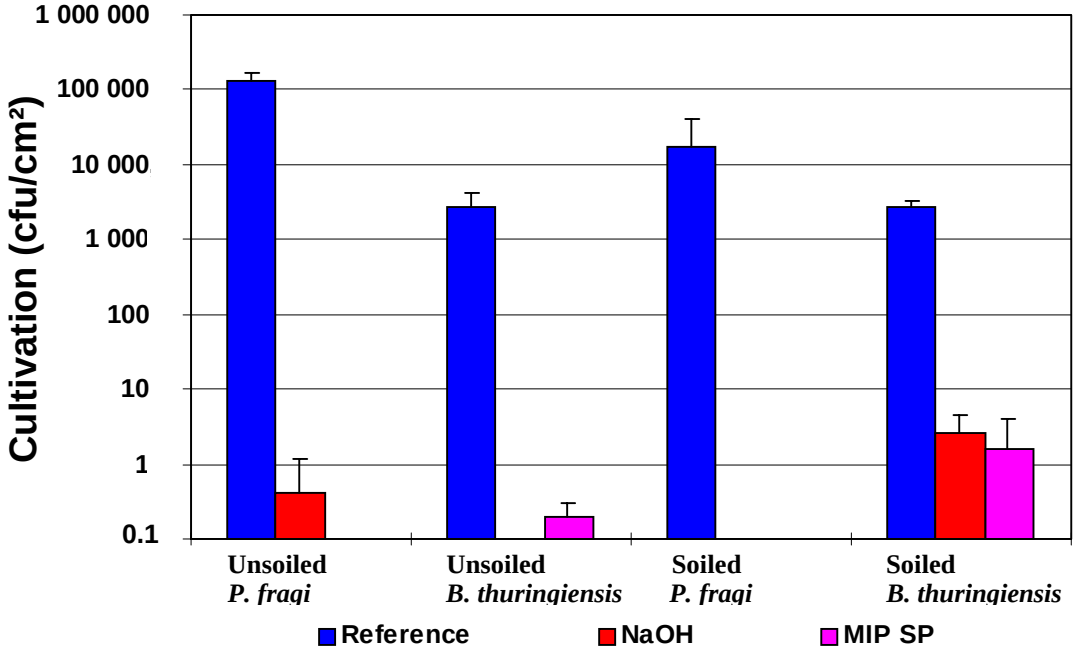

Figure 5. The results of swabbed surfaces using conventional cultivation showed that some living cells were present after the treatments with both sodium hydroxide (NaOH) and potassium hydroxide (KOH) based MIP SP.

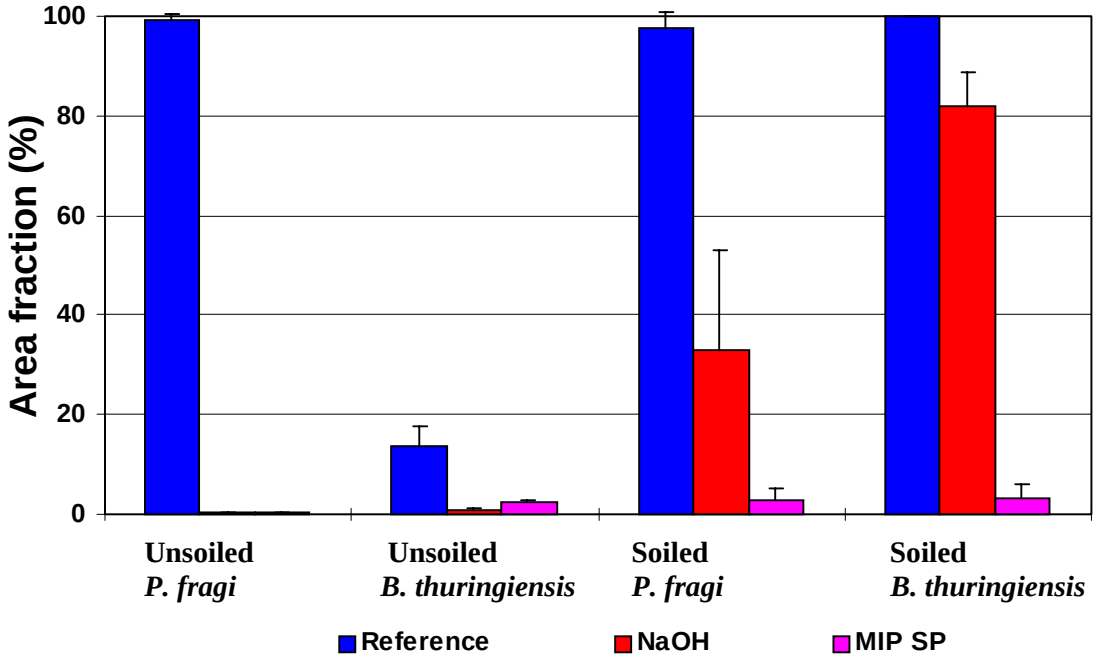

Figure 6. Epifluorescence microscopy performed directly on surfaces after staining with acridine orange, showed that the cleaning effect was more efficient with potassium hydroxide (KOH) based MIP SP than with 1% sodium hydroxide (NaOH).

Moulding with TTC-agar

These results are difficult to interpret particularly in comparison with the results of the other methods used in this experiment. However, it was evident that a treatment with only sodium hydroxide was not a good cleaning agent for presoiled surfaces.

Impedance

Results from the impedance measurement are shown in Figure 7. The detection time of positive controls in all samples was less than 6 h and after swabbing it was less than 8 h. This was logical, because swabbing releases bacteria from the surfaces. The impedance measurement showed that the alkaline treatment with MIP SP was effective against *P. fragi* and *B. thuringiensis*, but not against spores of *B. thuringiensis*. Both sodium hydroxide and sodium hydroxide with StabiCIP EA added were ineffective against biofilms on unsoiled surfaces. The behaviour of *B. thuringiensis* spore suspension on surfaces was interesting, because none of the cleaning agents effected the spore suspension (results not shown).

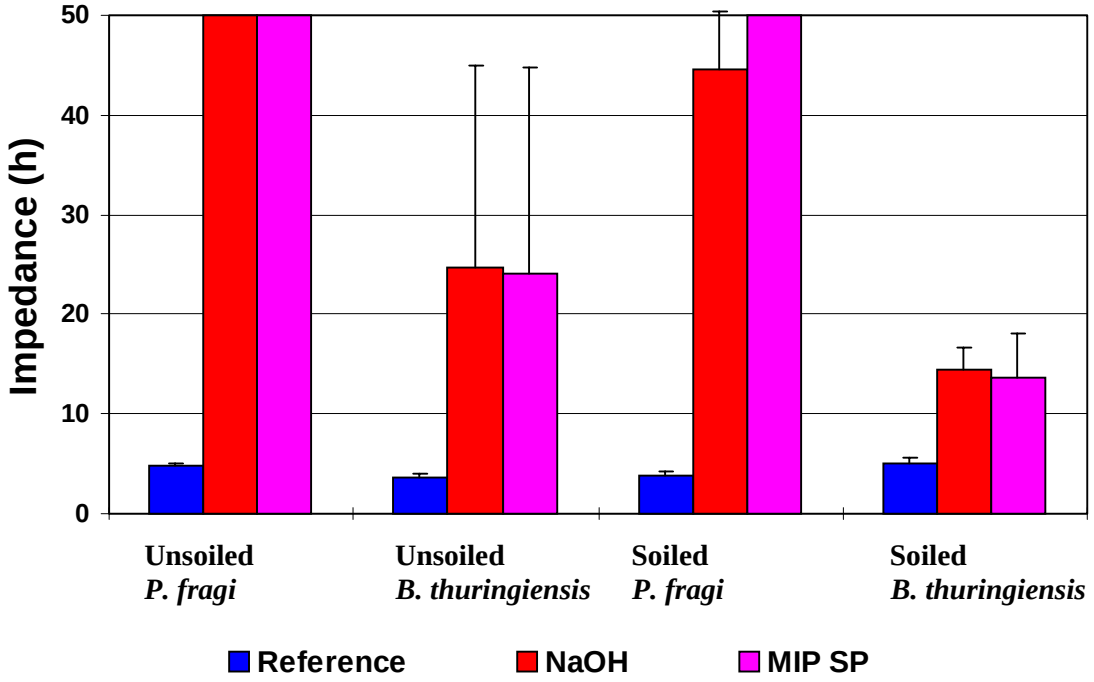

Figure 7. The impedance measurements were measured using coupons in the test cells. The results showed that none of the cleaning agents (sodium hydroxide and potassium peroxide based MIP SP) could remove B. thuringiensis *spores. Both cleaning agents showed to be effective against the* P. fragi *biofilm on presoiled surfaces.*

3.3 TESTING OF METABOLIC INDICATORS

3.3.1 Bacteria in suspensions

LIVE/DEAD stain stained alive cells green and dead cells red, while injured bacteria which could recover and reproduce were stained yellow or orange. The color difference between living and dead bacteria was clear and distinguishing this very easy, which can be seen in Figure 8. The stain faded rapidly, necessitating that preparations should be examined within the same working day to obtain reliable results.

LIVE/DEAD staining also stained the structure of biofilm. The amount of biofilm could thus be easily analysed. Metabolic activity was not possible to investigate directly from the surface with this stain, because the entire 'net' structure was stained green, which could be interpreted to mean that all bacteria were alive. The separate cells were stained red in both control and treated biofilm samples, which could be interpreted to mean that the bacteria were dead. While using this direct staining manner it was also impossible to determine whether or not cells were present inside the 'net' structure.

The amount of viable bacteria actually obtained for suspension was similar to the theoretical amount of cells. According to the results of swabbed biofilm bacteria of both *P. fragi* and *L. monocytogenes*, 80 ± 10% of the cells were viable in 2-d-old biofilm. The results obtained by biofilm staining with LIVE/DEAD stain showed that viable cells were found in only 1% of the analysed area due to interference with polysaccharides. The interpretation of results was very difficult, even when using a color camera and a color monitor. The shades of green (living cells) and red (dead cells) were similar in the image analyser, resulting in misinterpretations.

According to the results 72 ± 6% viable cells were present in *P. fragi* biofilm grown on unsoiled stainless steel surface and 85 ± 9% viable cells in biofilm grown on NORDFOOD-soiled stainless steel surface. *Bacillus thuringiensis* grew similarly on both soiled and unsoiled surfaces; the percentage of viable cells was about 50 ± 5%. The corresponding values for *L. monocytogenes* biofilm were 67 ± 9% viable cells on unsoiled surface and 83 ± 4% viable cells on soiled surface. Fewer viable cells were present in the mixture of *P. fragi* and *L. monocytogenes* than in samples of either *P. fragi* or *L. monocytogenes*. The percentage of viable cells in the mixture grown on unsoiled surfaces was 58 ± 8% and on soiled surfaces 81 ± 5%. These viability percentages of *P. fragi* and *L. monocytogenes* were similar to the results obtained previously in this study for these strains. Similar results were obtained with both LIVE/DEAD and CTC-DAPI stainings.

3.3.2 Biofilm bacteria

CTC stained a fluorescent red dot in living cells, while DAPI stained all the cells fluorescent green. A photograph of a 2-day-old biofilm of *P. fragi* is shown in Figure 9. Since both living and dead cells fluoresced, the total number of cells could be counted with image analysis. This was done, because only a black-and-white camera was available. The numbers of red dots (living cells) or green cells (dead) were counted manually depending on which were less.

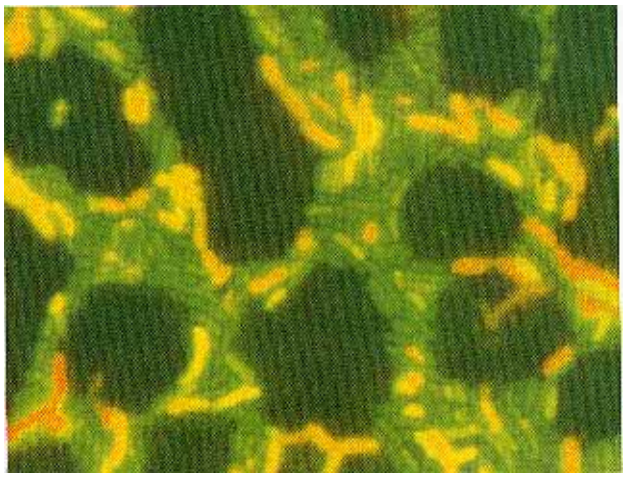

Figure 8. A suspension of living Pseudomonas fragi stained with LIVE/DEAD stain. The samples were prepared by mixing 1 ml of suspension and swabbed biofilm with 6 µl LIVE/DEAD stain and incubated for 15 min in the dark at room temperature.

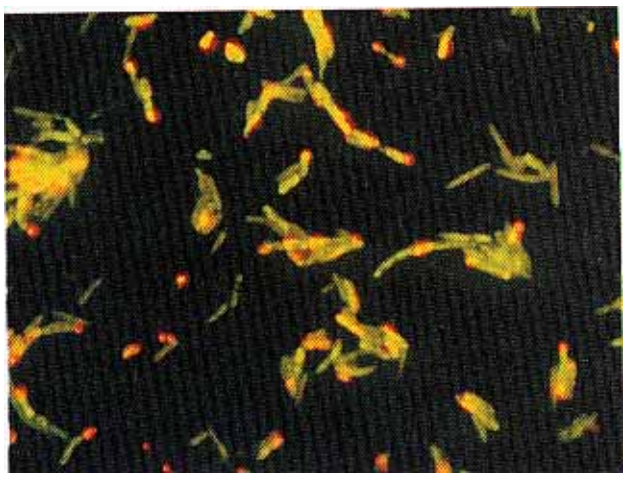

Figure 9. A biofilm of living Listeria monocytogenes stained with CTC and DAPI. The stainless steel coupon with biofilm was stained with 2 ml of 5 mM CTC for 2 h at 37 ± 1° C. The stain was fixed with 2 ml of 5% formaldehyde for 5 min at room temperature and counterstained with 2 ml of 1 µg ml^{-1} DAPI for 3 min.

According to results of the swabbed biofilm bacteria of both *P. fragi* and *L. monocytogenes* stained with CTC-DAPI, about 70 ± 10% of the cells were viable in two-day-old biofilm. The viability percentage obtained in all the swabbed biofilm bacteria samples was lower than the theoretical percentage of viable cells. These findings corresponded to those of Bovill *et al.* (1994) and Rodriguez *et al.* (1992). This appeared to be a more realistic result than the one obtained by LIVE/DEAD staining, indicating that only the control sample was below the theoretical value.

The CTC was fixed with formalin in modified CTC-DAPI staining to prevent fading. Fixation enabled the investigation of biofilm samples, because the fluorescent red dots in living cells were more stable than without fixation. In *L. monocytogenes* biofilm samples fixation did not prolong the time before the red dot vanished, but the dots showed much better fluorescence than the rest of the cell, making the differentiation of living and dead cells possible.

In modified CTC-DAPI staining, the total number of cells was counted with image analysis and the number of living cells manually. Some red dots were present in the biofilm structure, which may have caused errors in counting the total number of cells. The program used counted each fluorescent dot as a cell; thus these extra dots made the total number of the cells greater than in actuality.

3.4 TESTING OF NOVEL SWABBING SOLUTIONS IN CULTIVATION

The cultivation results of novel swabbing solutions tests are shown in Figures 10 and 11. Test surfaces were either unsoiled (Figure 10) or presoiled (Figure 11) with NORDFOOD-soil and covered with biofilms of *P. fragi* and *B. thuringiensis.* The measurements were carried out both before and after CIP-cleaning with different alkaline cleaning solutions. Surfaces with biofilms which were not CIP-treated were used as references. The remaining biofilms were detected by swabbing the surfaces with swabs moistened in both saline and detergent solutions. The main components in the detergent solutions tested were surfactants, alcohols together with abrasive chemicals and enzymes together with abrasive chemicals.

In this experiment, results from different swabbing techniques were quite similar, but some trends could be detected in cultivation results. The main differences that could be noted in the CIP procedures performed were that soiled surfaces were more efficiently cleaned using a CIP agent containing EDTA. This aspect was clearly detected using swabs moistened with detergent solutions containing surfactants or enzymes and abrasive chemicals. The same detergent solutions also improved the detection of *B. thuringiensis* spores remaining on the surfaces.

The area fractions of biofilms remaining on both unsoiled and presoiled stainless steel surfaces detected with epifluorescence microscopy confirmed the results of cultivation (Figures 12 and 13). The spores of *B. thuringiensis* withstood CIP cleaning better than did biofilms of *P. fragi*. In addition, small differences between swabbing techniques could be observed from the results of unsoiled surfaces where

swabs moistened with detergent solutions containing surfactants or enzymes and abrasive chemicals had removed most of the biofilm. The remaining area of biofilm for *P. fragi* (unsoiled surfaces) after peptone saline swabbing was about one half of the value before swabbing and after enzyme swabbing about one tenth.

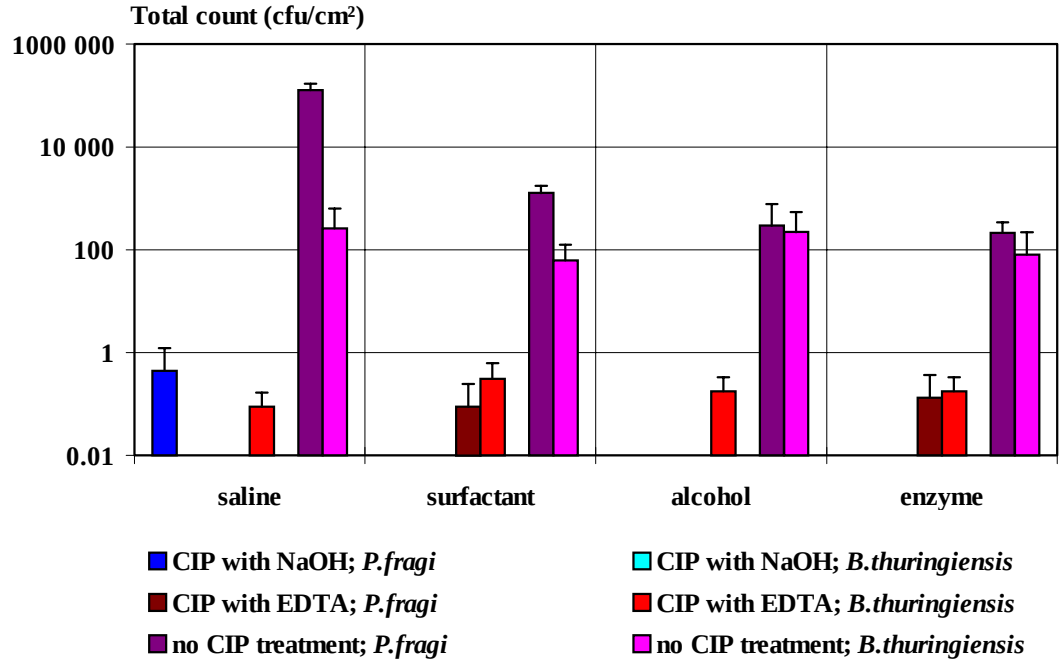

Figure 10. The cultivation results of novel swabbing solution tests using unsoiled surfaces covered with biofilms of Pseudomonas fragi *and* Bacillus thuringiensis *both before and after various CIP-cleaning procedures.*

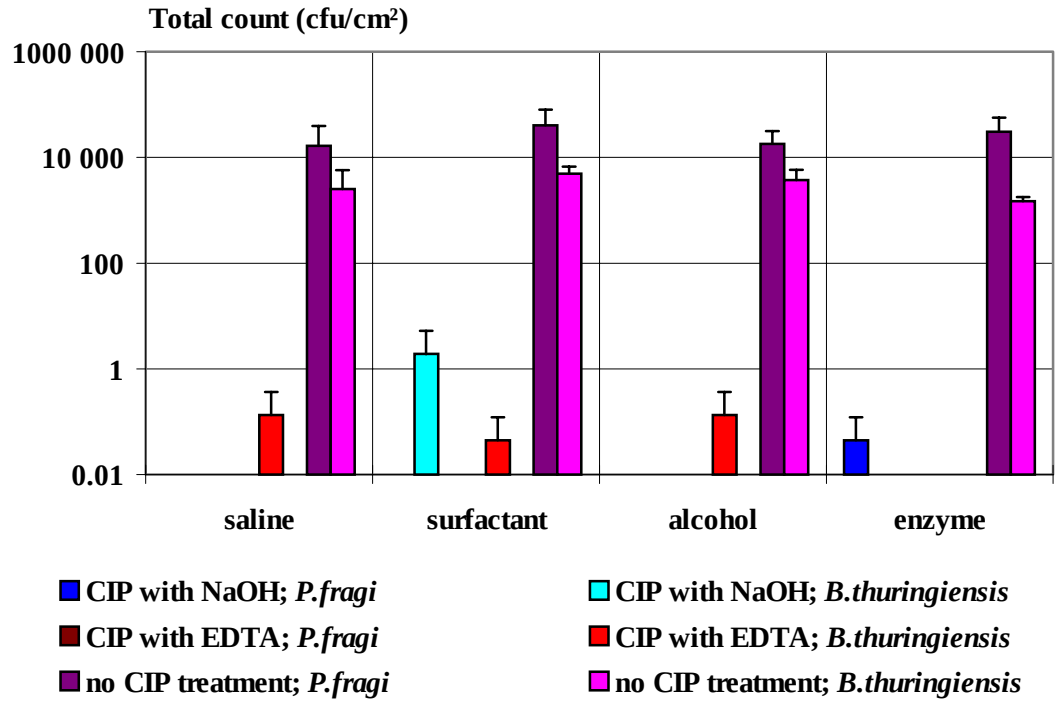

Figure 11. The cultivation results of novel swabbing solution tests using presoiled surfaces covered with biofilms of Pseudomonas fragi *and* Bacillus thuringiensis *both before and after various CIP-cleaning procedure.*

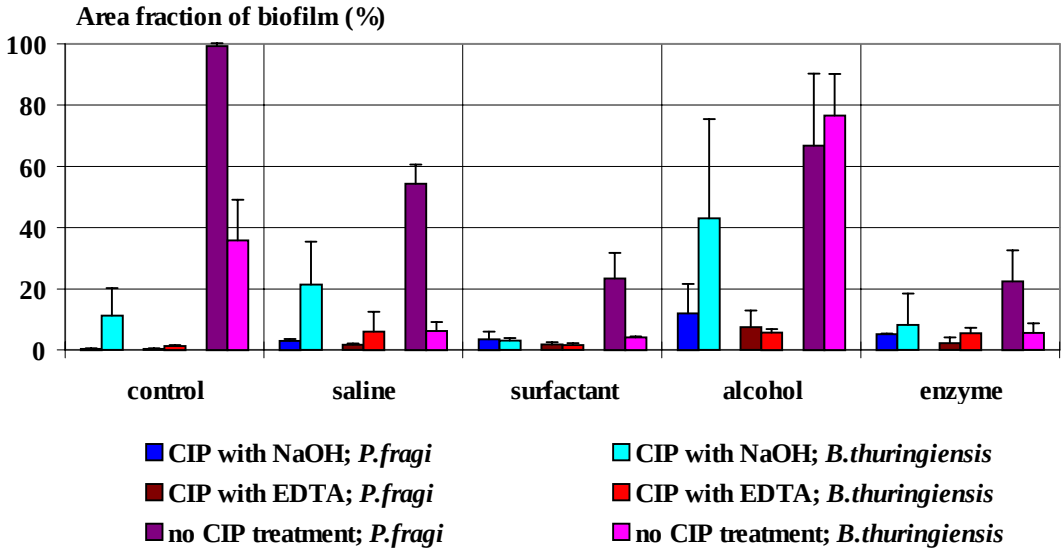

Figure 12. The results of epifluorescence microscopying of novel swabbing solution tests using unsoiled surfaces covered with biofilms of Pseudomonas fragi *and* Bacillus thuringiensis *both before and after various CIP-cleaning procedures.*

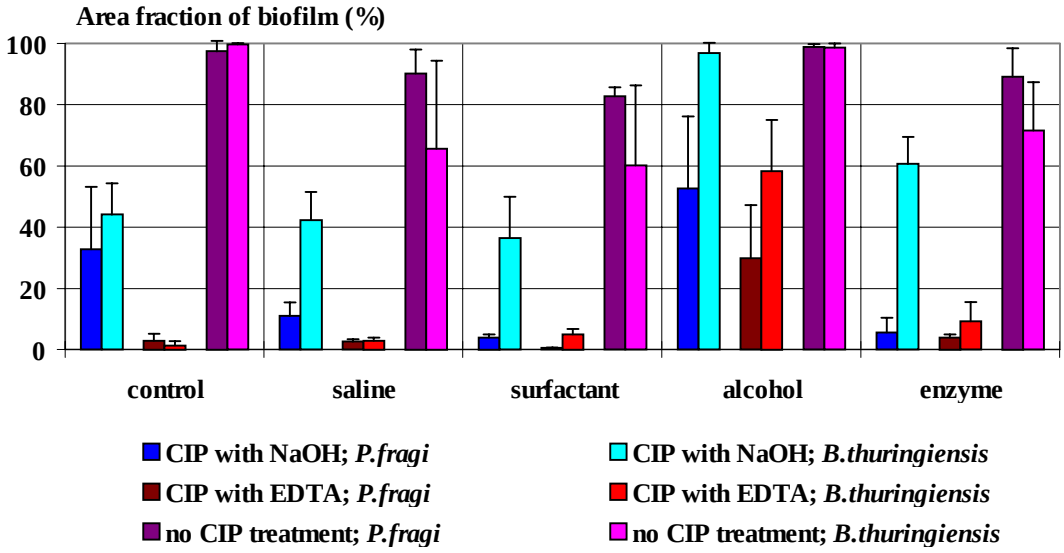

Figure 13. The results of epifluorescence microscopying of novel swabbing solution tests using presoiled surfaces covered with biofilms of Pseudomonas fragi *and* Bacillus thuringiensis *both before and after various CIP-cleaning procedures.*

The results from the evaluation of detergent solutions for sampling in industrial environment are given in Figures 14 and 15. The use of the different detergent solutions in evaluating the cleaning efficiency of open process surfaces was carried out at nine check points. Only two of these checkpoints were slightly contaminated. At these points the product was in contact with the process surface. The spraying of the sampling solution seemed to enhance release of microbes from the surface. According to the cultivation and contact plate results the alcohol based solution was the most efficient releaser of microbes in processes handling fatty products.

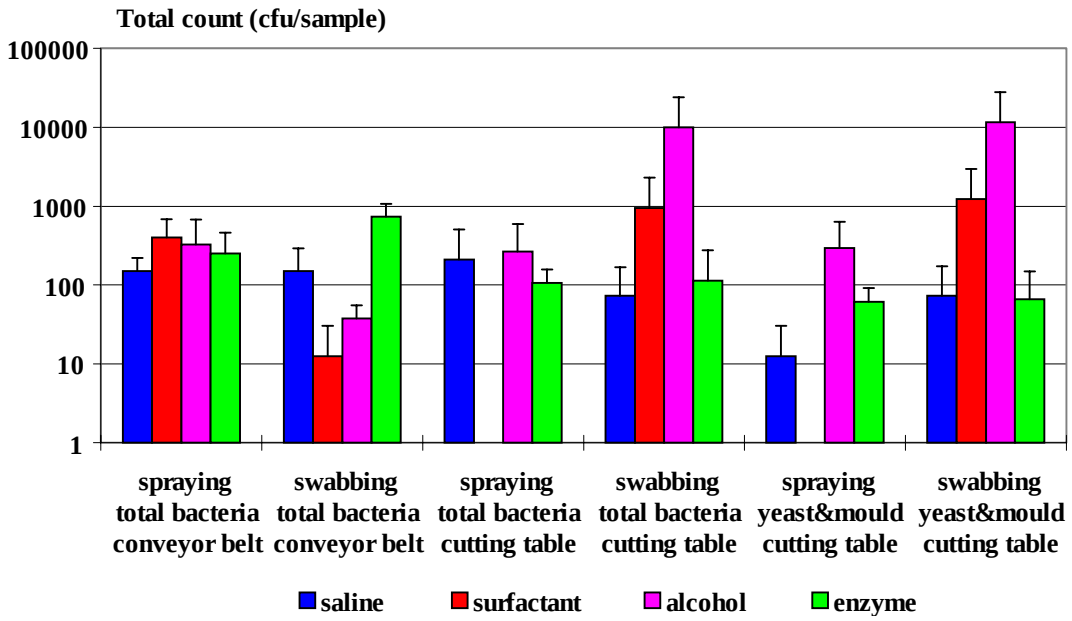

Figure 14. The evaluation of detergent solutions for sampling in industrial environment using swabs moistened with detergents and dry swabs with detergents sprayed on surfaces. Samples from two different process surfaces were cultivated on agars both for total bacteria and for fungi.

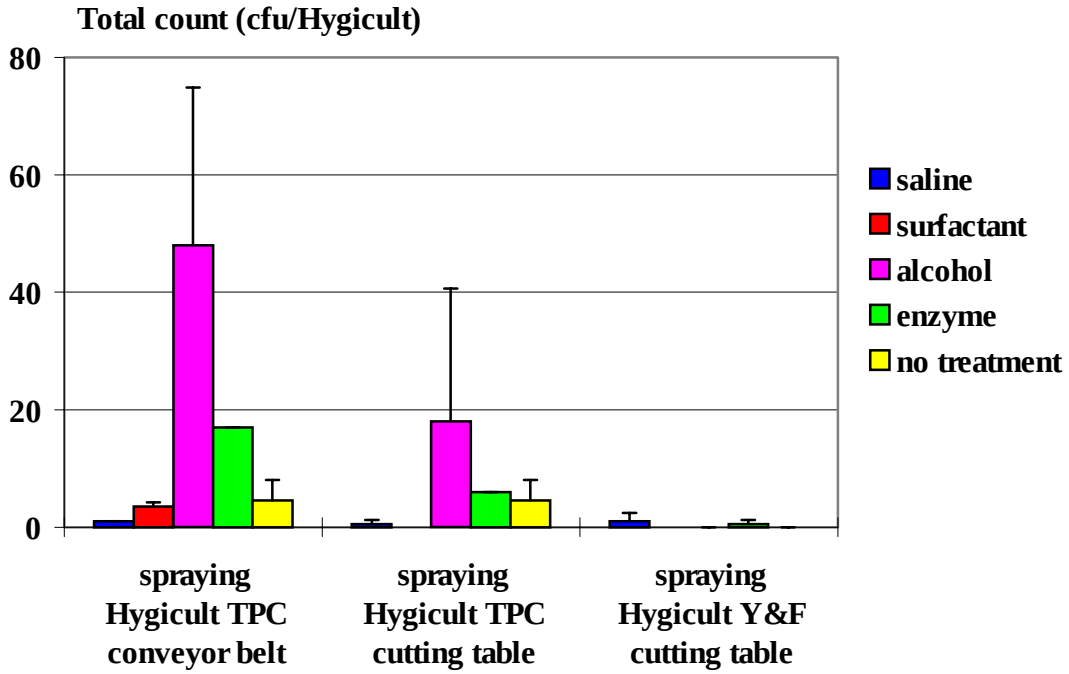

Figure 15. The evaluation of detergent solutions for sampling in industrial environment using detergents sprayed on surfaces and sampling with contact agar slides: Hygicult® TPC (Total Bacterial Count) and Hygicult® Y&F (Yeasts & Fungi).

3.5 TESTING OF DISINFECTION IN INDUSTRIAL SCALE

The results from the first experiment showed that the weekly averages seemed to increase with increasing frequency of fogging. The period without fogging was too short to obtain reliable information on the range of natural variations for the different samples and microbes. The results may therefore represent random variations, and the apparent negative correlation between frequence of fogging and number of microbes only a coincidence. The remaining fungal counts after fogging are presented in Figure 16. If the results really demonstrated a negative effect of fogging the reason could have been the following:

- the fogging equipment did not work properly and the concentration of the disinfectant was too low (possibly througout the whole period) and
- the fogging increased the humidity in a room which is normally dry, which provided good growth conditions for the microbes.

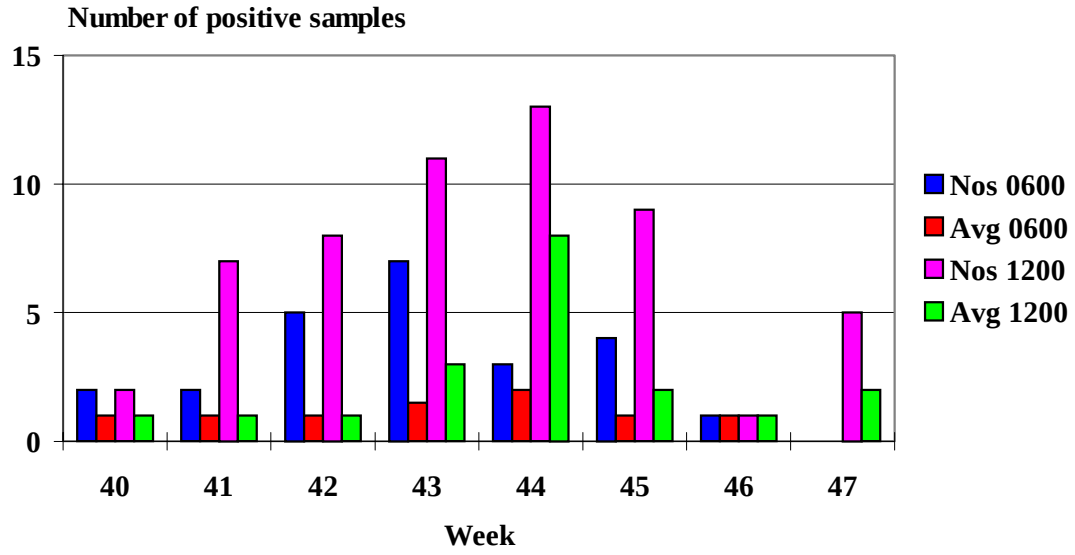

Figure 16. The results show the amount of fungal (yeast and moulds) cells in the air as number of positive samples and average numbers per week. The sampling was performed using an air sampler (320 l/sample except for weeks 40 and 41 when 180 l/sample was taken)

The evaluation of microbiological results in the other dairy, where fogging was carried out, was uncertain due to the relatively few samples compared to the large variations in the material. Significant reduction of non-lactic bacteria was found in 6 of total 12 sampling points with both of two different statsistical methods. Coliforms at 2 p.m. in two sampling points had a significant increase in the period with fogging. The changes in microbiological standard did not seem to have improved as a result of fogging. However, the changes in microbiological levels were small and they could have been caused by random variation i.e. due to other factors than fogging. The results were not clear, but the main impressions were:

- the use of fogging did not produce any clear improvement in the microbiological standard of the air or on the surfaces,
- the total count in air was not changed except from a minor increase during the fogging period,
- moulds/yeasts on surfaces did not occur at 7 a.m. and there were no significant changes in the results obtained at 2 p.m. and
- the number of coliforms on surfaces was generally low, but at 2 p.m. there was a minor increase at all sampling points during the fogging period.

The experiment with inoculated coupons was influenced by the malfunction of the fogging equipment. The results showed that bacteria on coupons, which were placed so that the fog reached the samples, were killed by the disinfectant. Coupons located under e.g. tables had similar counts to those on controls. The effect of the rinsing period was also insufficient, which meant that the disinfectant was not removed but only diluted. In such case the time of disinfection was prolonged, additionally the concentration of the disinfectant would increase due to evaporation of water.

4 CONCLUSIONS

4.1 DISINFECTANT TESTING IN SUSPENSION AND ON SURFACES

The suspension test described in prEN 1276 can give information about a disinfectant, and how it's effect is influenced by protein and hard water. In dairies, bacteria can exhibit increased resistance due to attachment followed by growth on wet surfaces. Therefore, suspension tests should be followed by practical evaluation tests simulating dairy environment.

Of the standard strains, *P. aeruginosa* was the most difficult to kill. Of the four test strains representing bacteria of concern to the dairy industry, *S. infantis* was the most difficult to kill. Endospores were also resistant to disinfectants, and the recommended in-use concentration intended for reduction of vegetative bacteria, should not be expected to apply for endospores. The disinfectant efficiency using 80% of the lowest recommended concentration in the prEN 1276 differentiated well between the disinfectants at the described test conditions.

The surface test was propably more stringent than the suspension test and it gave information on how disinfectants acted on contaminated surfaces. The surface test is therefore an alternative and an additional method to the suspension test for evaluating the efficiency of disinfectants. However, more work needs to be done to test the precision of the method.

4.2 CLEANING OF SURFACES IN OPEN SYSTEMS

The aim of the experiment in cleaning of open systems was to develop microbial methods for the detection of biofilm and bacterial cells left on surfaces after cleaning and disinfection performed. Conventional cultivation, combined with impedance measurements together with image analysis and CTC-DAPI staining gave results that were comparable, complementary and enabled a total evaluation of both the removal of biofilm and the killing of bacterial cells. The results indicated that the low pressure application system was not effective in removing all the biofilm unless the foam agent itself was effective. The efficiency of the foam agent was dependent on its ability to remove biofilm from the working surface combined with the ability to kill the bacteria present in the biofilm. The foam agent must therefore remain in contact with the surface for a sufficient length of time without drying.

The results of the tests using CTC-DAPI staining showed that the effects of all four cleaning procedures were quite similar. Treatment with Topax 12 combined with Oxonia Aktiv or Virkon S appeared to be somewhat more effective than the treatment with Topax 12 alone. These results thus indicated that the disinfectants exerted a surprisingly low effect on cleanability. The results obtained by cultivation showed a greater difference between the cleaning methods, which partly may be explained by the stress caused by the cleaning and disinfective agents.

4.3 CLEANING OF SURFACES IN CLOSED SYSTEMS

The aims of the experiment carried out using closed systems were to evaluate different CIP procedures and to develop microbial swabbing methods for the detection of biofilm and bacterial cells left on surfaces after CIP treatments. Conventional cultivation, image analysis and impedance gave comparable results and complemented each other. The concentration of the cleaning agents used did not destroy all the bacteria growing in biofilms on surfaces.

The cleaning effect on unsoiled surfaces was better than on soiled ones. None of the treatments removed all of the biofilm, and low numbers of viable bacteria still remained on the coupons. The spores of *B. thuringiensis* were more firmly attached compared to biofilm. Furthermore, spore-forming foodborne bacteria have high heat and chemical resistance. The results provide interesting information concerning the methology of hygiene testing. Formation of slime on soiled surfaces may lead to very high area values even in cases where the cultivated cell number was relatively low.

The efficiency of the cleaning agent is dependent on its ability to remove biofilm from the working surface, combined with the ability to kill the bacteria present in the biofilm. Chelating agents in the cleaning solution seemed to enhance the removal of biofilms from processing surfaces but frequently left some living bacteria on the surfaces. Treatments with sodium hydroxide containing StabiCIP EA and MIP SP were more efficient in removing

biofilm than sodium hydroxide and sodium hydroxide with SU560 (results not shown). The use of various methods and microbes in cleaning procedures at pilot scale helps to interpret the results of cleaning efficiency achieved in process scale with cleaning agents and disinfectants available on the market.

4.4 TESTING OF METABOLIC INDICATORS

The present results showed that the LIVE/DEAD staining procedure offered a rapid, easy, and reliable method for investigation of suspension and swabbed biofilm bacteria, although it did not work with biofilm attached to surface, due to interference with polysaccharides of the biofilm matrix and stain.

CTC-DAPI combination also offered a reliable method for suspension and swabbed biofilm bacteria investigation, in addition, modified CTC-DAPI staining can be used to stain the biofilm directly on the surfaces. Formalin fixation of the CTC enabled the image analysis of biofilm bacteria attached to the surface. The incubation period of 2.5 h was longer than the incubation period of the LIVE/DEAD stain, which was 15 min. The results of suspension and swabbed biofilm bacteria were as reliable as the results obtained by LIVE/DEAD staining.

According to the results obtained, ATP measurement was not a reliable metabolic indicator for biofilm and swabbed biofilm bacteria. The number of colonies in 2-d-old biofilm was not sufficient to achieve reliable results with the ATP measurement, which appeared to be more reliable for bacterial suspension than for biofilm bacteria.

4.5 TESTING OF NOVEL SWABBING SOLUTIONS IN CULTIVATION

The results of cultivation in comparison to microscopy showed that sampling from a surface was improved when using swabs moistened with detergent solution containing surfactants or enzymes together with abrasive chemicals. Despite of cell injury during the CIP procedure, which made cells very sensitive to the toxic properties of detergent solutions, the sampling technique using novel detergents showed to be more effective than traditional sampling. The microscopic and impedance assessments of swabbed surfaces showed that some degree of biofilm remained on the surface. Detergent solutions suitable for sampling should be chosen case by case taking into consideration the process conditions, growth conditions for the biofilm and the level of sensitivity needed.

4.6 TESTING OF DISINFECTION IN INDUSTRIAL SCALE

Controlled experiments with fogging on an industrial scale were carried out at two cheese producing dairies. Samples from air were analysed for total fungal and bacterial counts. The surface samples were analysed for fungal, coliform and non-lactic bacterial counts. Sampling was carried out both before the start of production and during production. The

studies in both dairies showed clearly the need for thorough control and following up of fogging systems. Critical points to be checked related to the quality and amount of the fog produced, the concentration of the disinfectant and also the effect of the rinsing. Malfunctions may be caused by varying pressure of the water, faulty mounting and adjustment of the system, clogged filters etc. Neither of the trials showed clear positive effect of fogging on the chosen microbiological parametres. If the apparent negative effects were representative and the microbes had increased with fogging, this could be explained by malfunction of the system, increased humidity and too low concentration of the disinfectant. Another possible explanation could be that the results were just incidental and that the natural occuring variations were too big and the analysis too few to draw any conclusions on the effect.

The effect of the rinsing period in the trials was questioned. Visual control gave the impression that the amount of water was far from sufficient to remove the disinfectant from horizonal surfaces. Insufficient rinsing is against public regulations. On the other hand it will prolong the period of killing effect and therefore improve the result of the disinfection. In the trials all the chosen control points on surfaces were critical points for good hygiene. Several of them were given special attention in the standard cleaning procedures which also included extra disinfection. Despite this and in spite of the fogging, the results from the analysis of these points varied from "zero" one day to ”overgrown” the following day. It is clear that such variations do not occur as an effect of the fogging system. The variations described above are the main problems when testing on an industrial scale. The challenge is to select the best and most representative points for testing, to select the right parametres for analyses and to keep all unwanted variations as low as possible. It would be necessary to run a test for a longer period than in this project. The difficulties connected with investigating fogging at industrial scale, clearly demonstrate the need for neutral and controlled tests in pilot scale.

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CLEANING AGENTS AND DISINFACTANTS USED AT VALIO DAIRIES 1993

Manufacturer/distributer	**Cleaning agents and disinfactants (amount/a)**
Kemira Ltd., Algol Ltd., Etek Ltd.	Nitric acid 60% (2 786 700 kg/a)
Kymi Ltd., Nokia Ltd. Veitsiluoto Ltd. Kemira Ltd. Kymi-Strömberg Ltd.	Caustic soda 100% (2 400 200 kg/a)
Farmos-Yhtymä Ltd.	Amisept, Aspi, Boxan,Caporit, Capo tab, Fet 34 Erlik DVS, Finol, Finosept, Fet 22 Hohta, Ipasept, Jama, Kloriitti-Forte (51 900 kg/a), Kope, Fet 23 Laatikonpesu, Fet 40 Loro (29 700 kg/a), Fet 63 Minikitka (17 100 kg/a), Neo-Amisept, Fet 42 Nora (287 100 kg/a), Fet 41 Nora, Fet 30 Painepesu, Pesetti 10, Pesuneste L, Pintty, Fet 43 Super-Nora (109 400 kg/a), Fet 11 Super-Tisko (58 800 kg/a), Tarmo, Fet 45 Teho-Sensol, Fet 50 Tero (19 300 kg/a), Fet Tervi, Timil, Tisko (25 400 kg/a), Fet 19 Triosept, Fet 44 Vaahtosensol, Fet 29 Vaahtosept, Fet 25 Vape
Suomen Henkel Ltd.	P3-asepti oxonia, P3-asepto, P3-CIP, P3-stabilon CIP, P3-elko (15 100 kg/a), P3-Fd rasvanpoistojauhe, P3-ferisol, P3-FPC, P3-FPC foam, P3-galvaclean, P3-grato 10, P3-hypo (18 400 kg/a), P3-juho, P3-kirsti (18 600 kg/a), P3-luboklar 60, P3-moni, P3-neste desta, P3-neste seta, P3-oxonia aktiv (49 100 kg/a), P3-PE 4, P3-rik, P3-seta, P3-super kepe, P3-system plus, P3-T 1166, P3-tensol, P3-topax 91, P3-topax LP, P3-topax 18, P3-topax 56, P3-topax 66, P3-trimeta 33, P3-triquart, P3-ultrasil 10, P3-ultrasil 11, P3-ultrasil 75, P3-vaahdonesto K, P3-oxonia vetyperoksidi 35% (17 900 kg/a)
Suomen Unilever Leverindus Ltd.	Alvo, SU-333 desipak, SU-156 jyty (166 300 kg/a), SU-159 kierto (16 500 kg/a), SU-350 kloori, SU-319 kvatti, SU-937 plast, SU-927 primo, SU-932 triple
Diversey Ltd.	Dicolube 2000 H (14 800 kg/a), Dicolube TP, Dicocip 99, Divopan, Divoschaum ML, Plastren
Veitsiluoto Ltd.	Sodium hypochlorite
Kymmene Ltd. Nokia Finnish Chemicals Ltd.	Oulu 920

CLEANING AGENTS AND DISINFECTANTS USED IN NORWEGIAN FOOD INDUSTRY[1]1996

Manufacturer/distributer	Cleaning agents and disinfectants
Aco Norge A/L Arrow Chemicals	Arrow Acifoam, Arrow Alkafoam 8, Arrow Alkafoam 20, Arrow Chloroclean, Arrow Chloro Clean m/skum, Arrow FPC, Arrow Germ Free, Arrow Heavy Duty Handcleanser, Arrow Hydroclean Bactericidal, Arrow Hyposan, Arrow Kassevask, Arrow Sanitiser QAC, Arrow Seafood Process Cleaner, Arrow Super Clean Consentrate, Arrow Syrevask
Arcon A/S	Alkaklor, Alkaren 45, Alkadan, Alkarøk, Arklor, Autoshampoo, Betane, Combiren, Combiren m/skum, Combisyre, Danakas, Danapluss, Danaskum, Danazur, Detersept, Flytende Isoren, Flytende Manuren, Flytende maskinoppvask, Flytende Novakombi, Flytende Novasyre, Formvask, Fosfatfri Aciren, Meierisåpe, Novaclean, Novakombi, Novasyre, Oxidan, Oxidan Ekstra, Ro-Dan Acid, Ro-Dan 92, Ro-Dan Pluss, Ro-Ten Alka, Ro-Alka Zym og Vognvask 1
Basol Norge AS	Basol 333, Basol CC, Basol DS 3, Basol F-110, Basol F-222, Basol Jetwash, Basol LDC, Basol M, Basol Quantum, Basol Superkraft, Ocean 719, Ocean 725, Ocean 729, Ocean 735, Ocean 921, Ocean 923, Ocean 926, Ocean 929, Ocean 931, Ocean 934, Ocean 938
Certified Laboratories A/S	Aqua Sol 20/20, Certisuds Plus, Colleen, Germa Cert Plus, Lift off, Rask Plus, Sure Flow Plus, Well Done
Chem-Tech Norge A/S	All-Rens, CT-Alkalisk Skumrens, CT-Surt skumrens, Desi-Fix, NMI
Chemsearch	Chemsearch Consentrate Extra, Dismiss, Everbrite Extra, ND 165, Shiny Side, Strong Arm, Swoop Extra, Tempest
Chemtrust AS	Trust C-Clean, Trust C-Clean Foam, Trust Asyr-Clean RWS, Trust Asyr Foam, Trust Bleach, Trust Max, Trust Safe, Trust Germ Free, Trust Jod, Trust Foam Power
Lilleborg A/S	Addi-11F SU 929, Addi-14, Addi-7, Addi Gel SU 628, Addi Gel SU 625, Addi Kraft SU 930, Addi Røkeovnsvask, Addi SU 928, Addi SU 727, Addi SU 935, Addi-K, Addiquat SU 321, Additive 810, Additive SU 560, Alkasterk 12, Alkasterk 140, Alkasterk 150 S, Alkasterk 170, Antiskum SU 773, Antiskum SU 971, Båndrens SU 846, Båndrens SU 847, Båndrens SU 853, Desinfekt 900, Desinfekt 951, Desinfekt SU 388, Jodocid, L-51, L-52, L-53, Sterivask 702, Sterivask SU 328, SU 118, SU 156 Climax M,

Manufacturer/distributer	Cleaning agents and disinfectants
Lilleborg A/S cont.	SU 159 Climax A, SU 192 Climax P, SU 328 Climax K, SU 357 Climax TK, SU 470 Climax S, SU 475 Climax SF, SU 477 Climax NFF, Sumadesi 1, Sumadesi 2, Sumagrov, Sumakraft, Sumaovn, Sursterk 612, Titan Hygiene SU-358, Titan Rinse SU-918, Titan SU 160, Titan Ultraclean SU-937
Delta Norge A/S	Delta Aluminiumsvask, Delta Chlor, Delta Desinfisering, Delta Jod, Delta Kassevask, Deltafoam 2
Diversey A/S	Dicolube CT, Dicolube RS148, Divocip 88, Divocip 164, Divosan Aktiv Plus, Divoskum 84, Divoskum 140, Divoskum 311 CL, Tik-Et
Electrolux Euroclean A/S	Eurodesinfect 100, Eurodesinfect 304, Euroskum, F44R, E204S, HD-Spesial, HD-Vask, Kassevask, Klorskum, Sirkulasjonsvask A, Proffs Combi
Freyasdal Fabrikker A/S	F.G.40, Fawa Alkalisk Sirkulasjonsvask, Fawa Desinfect, Fawa Grovrengjøring, Fawa S-3, Fawa Sur Sirkulasjonsvask, Fawa Universalrengjøring, Fawa Surt Sanitær, R-45 Røkeovnsrens, R-46 Røkeovnsrens, Skumvask A 21-X, Skumvask K
Gras Produkter A/S	Gras Des-7, Gras Des-11, Gras Kraft Des, Gras Universal, Gras Universal Plus, Gras Universal Sterk
Henkel-Ecolab A/S	Alcodes, Asepto, Asepto Flytende, Ancep BPC, Ancep CIP, Ancep TOD, Clint CA 157, Diskal SK, Duett K, Duolit SK, Flytende 148, Horolith BSR, Horolith DES, Horolith LA, Horolith MK, Horolith TR, Horolith V, Horolith VN, Horolith 283, Hypokloran SP, ISS Alkali 21, 1SS Alkali 25, Luboglide LF, MIP Alu, MIP FL, MIP Zentra, MIP Cip, Oxonia, Oxonia Aktiv, Oxonia Aktiv S, Prevafoam PB, Pripan, Stabilon AL-Flytende, Stabicip AD, Stabicip NA, Stabicip EA, Stabilon MB, Steril, Super LA, TP 12, TP 16 SK, TP 17 FR, TP 18 SK, TP 19 FR, TP 21, TP 32, TP 55 SK, TP 58, TP 66, TP 68, TP 95, TP 99, Trimeta Sur N, Trimeta OP 157, Trimeta 33, Trimeta 60, Triquart, Triquart AP, Trital FS, Ultrasil 10, Ultrasil 11, Ultrasil 25, Ultrasil 41, Ultrasil 50, Ultrasil 53, Ultrasil 56, Ultrasil 60 A, Ultrasil 70, Ultrasil 72, Ultrasil 75, Ultrasil 91, Ultrasil 93, Ultrasil 96, VR 1250-2, Z
Kärcher A/S	RM 22 Super Kraftvask, RM 25 Sanitærvask, RM 31 Super Kraftvask, RM 33 Røykhartsfjerner, RM 81 Kraftvask, RM 732 Desinfekt Alkalisk, RM 735 Desinfekt nøytral
Krefting & Co. AS	Selaclean AF 2000

Manufacturer/distributer	Cleaning agents and disinfectants
Laporte A/S	Kleencare AN144, Kleencare AN149, Kleencare CF300, Kleencare DS603, Kleencare DS680, Kleencare NF400, Kleencare NF420, Kleencare NF421, Kleencare NN479, Kleencare NN499, Kleencare NNM472, Kleencare SF520, Kleencare SN545, Kleencare SN595
Norsk Chemi A/S	Lyso 3025, NC- 50, NC- Aktiv, NC- Allrent, NC- CL, NC - Desinfekt, NC- Glans og Tørremiddel, NC - kassevask, NC- Kraftskum, NC- Kraftvask, NC- Mask.oppvaskmiddel, NC- Oppvaskmiddel, NC- skumrens, NC- skumvask Ekstra, NCSkumvask m/klor, NC- Tank/Sirkulasjonsvask, NC - Tankvask
Occo Norway A/S	Desifin, E-G, Frisk, Glans, Kraft Rens, Safir Vask
Perfect Norge A/S	Perfect Game 13 Proteinrens, Perfect Game 85 Foamclean 7, Perfect Game 135 Røykovnsrens, Perfect Game 136 - Foamclean, Perfect Game 140 - Tankrens Alka, Perfect Game 139 - Multi C.l.P., Perfectoxid Desinfeksjon, Perfect Desinfect
Partner Farma A/S	Sterisol Virkon S
Remitek A/S	Remi Bakto 01, Remi Kombi 20, Remi Kombi 21, Remi Kraft 30, Remi Kraft 31, Remi Kraft 32, Remi Kraft 33, Remi Natur 2, Remi Skum 39, Remi Sur 50
Aksjeselskapet Sibi	Unisol 03, Unisol 07, Unisol 90
Skien Såpefabrikk	Grill-Vask, NM 120-A, NM-130-A, NM-150-S, NM-180-DK, NM-190-DK, NM-190MK, NM-200-Skum, NM-210-A, NM - 300-KA
Statoil Norge AS	Desinfekt SN 96, Kraftskum F422, RSW-Vask F441, Yrkesvask
Strøvelskemi Norge A/S	A 352, Alfacid

1) information given by TKVDN

RAPPORT ”SANITERING I MEJERI” ABSTRAKT

Projektet påbörjades med en genomgång av hurudanna kommersiella rengörings- och desinfektionsmedel som används i nordiska livsmedelsföretag. Baserat på förbrukning av och användningsområden för dessa rengörings- och desinfektionsmedel prioriserades ämnena med tanke på bruk i projektet. Variationer i testprocedurer och erhållna resultat för testning av nämnda ämnen underströk vikten av att harmonisera testningsförfaranden för användning i mejerier. Parametrar från skumrengöring av öppna processutrustningar och CIP-procedurer i slutna processsystem användes som grund för planering av experimenten i pilotskala. Experimenten omfattade också testning av desinfektionsmedel med mikrober både i suspension och på yta.

Konventionell odling av bakteriernas totala antal valdes som rutinmetod. Odlingen mäter de mikrober som kan bilda kolonier på näringsagarytan under de givna förhållandena. En infärgningsteknik av celler på processytor vidareutvecklades för mätning av rengöringseffekten och svabbning med nya provtagningslösningar utvärderades i både pilot- och processkala. Utvärdering av odlingsresultat i kombination med mikroskopiresultaten visade att provtagningen från ytor förbättrades när svabbarna fuktats med lösning innehållande ytaktiva ämnen eller enzymer och slipmedel. Biofilmrester och smuts som fanns på ytorna mättes med bildanalys av preparat, vilka färgats med akridinorange. Mikroskopering av preparat infärgade med både DNA-färg och metaboliska indikatorer gjorde det möjligt att skilja på levande och döda celler, som fanns på ytorna efter behandlingen. Dessutom användes impedans, kontaktagar, ingjutning med agar och ATP som mätmetoder i undersökningen.

Sex **desinfektionsmedel** utvalda av representanter från Valio Oy och Norske Meierier **testades** enligt test föreslagna av CEN (prEN 1040 och prEN 1276). Mikrober som testades var de i standardmetoderna givna mikroorganismerna samt fördärvande bakterier, patogener och sporbildare (endosporer), vilka förorsakar problem i mejeriindustrin. Inga skillnader mellan de olika desinfektionsmedlen kunde urskiljas i test utförda enligt prEN 1040 metoden. Stora mängder av störande ämnen ss. skummjölk och hårt vatten, som används i prEN 1276, reducerade desinfektionsmedlens effekt i varierende grad. *Staphylococcus aureus* och *Pseudomonas aeruginosa* var de mest resistenta mikroberna mot samtliga desinfektionsmedel. Bland de fyra ”industristammarna” var *Salmonella infantis* den mest motståndiga. Sporer av *Bacillus cereus* och *Bacillus thuringiensis* reducerades med mindre än 1 log-enhet i suspensionstesten. En användning av 80 % av den lägsta rekommenderade brukskonsentrationen skiljde väl på de använda desinfektionsmedlen under beskrivna testförhållanden i prEN 1276. Resultaten visade att det alkoholbaserade IPA 300 var det effektivaste av de undersökta ämnena. Försöken visade klart att test med strängare kriterier, i vilka praktiska förhållanden simuleras, behövs för att påvisa desinfektionsmedlens effekt i verkligheten. Resultat från yttest (CEN TC 216) visade att *Pseudomonas fragi* intorkade på ytan var mer resistent mot kemikalier än celler i suspension var.

Resultaten från **experiment i slutna system** visade att behandling med alkaliska rengöringsmedel innehållande kelaterande ämnen och kaliumhydroxid avlägsnade smuts och biofilm mer effektivt än enbart natriumhydroxid gjorde. Lämpligheten av rengöringsmedlen berodde på smutskonsistensen, bakterietypen och ytmaterialet. Resultaten från mikroskopering och impedansmätning av svabbade ytor visade att rester av biofilm fanns på ytorna efter samtliga rengörings-procedurer.

Resultaten från **experiment med lågtryck i öppna system** visade att behandling med starkt alkalina rengöringsmedel innehållande hypoklorit avlägsnade biofilm effektivare än det milda alkalina medlet, som rekommenderas för rengöring av annat än processytor. Bakterietalet på ytor utan smuts var lägre än på de med organiskt smuts behandlade ytorna. Avlägsnandet av biofilm från ytor m.h.a. lågtryck är ytterst beroende på skumrengöringsmedlets effekt. Skum-rengöringsmedlet bör vara i kontakt med ytan tillräckligt länge utan att torka in. Effektiviteten av medlet är beroende på både avlägsning av smuts samt biofilm och avdödning av alla på ytan kvarblivna bakterierna.

Målet med testing av **”fogging” eller total desinfektion i industriell skala** var att undersöka effekten på ytor vilka utsatts för desinfektionsmedel. Fogging kan definieras som ”kemisk desinfektion användande automatisk duschning med desinfektionsmedel i slutna rum”. Kontrollerade försök med fogging utfördes i två ostproducerende mejerier. Inget av försöken visade någon tydlig minskning i den mikrobiella belastningen. Experimenten vid de båda mejerierna påvisade klart nödvändigheten av en grundlig kontroll och uppföljning av proceduren. Kritiska punkter var kvaliteten på och mängden av dimman samt konsentrationen för desinfektionsmedlet och sköljningsprosessen. Otillräcklig sköljning späder enbart ut desinfektionsmedlet, varvid också rester blir kvar på ytorna. Dimningen ökar också på luftfuktigheten i rummet, vilket kan bidra till ökad korrosion och skador i elektrisk utrustning. Sammanfattningen av dimexperimenten visade att effekten kunde ifrågasättas speciellt i kritiska punkter och i torra utrymmen.

SAMMANFATTNING AV RAPPORTEN

Testing av desinfektionsmedel i suspension och på ytor

Suspensionstestet beskrivet i prEN 1276 ger upplysning om hurudan effekt ett desinfektionsmedel har och hur det påverkas av protein och hårt vatten. I mejeri-miljö kan bakterierna utveckla en förhöjd resistens som följde av att de fäst sig och vuxit på våta ytor. Det är därför viktigt att suspensionstesterna följs av en praktisk utvärdering, i vilken mejerimiljö simuleras.

Bland standardstammarna var det *Pseudomonas aeruginosa*, som var den svåraste att dräpa. Av de fyra teststammarna, som representerade s.k. problembakterier i mejeriindustrin, var *Salmonella infantis* den svåraste att avdöda. Endosporer var också resistenta mot desinfektionsmedel. Man kan inte förvänta sig att brukskonsentrationer som rekommenderats för avdödning av vegetativa bakterieceller kommer att bita på endosporer. Användning av 80 %-ig mängd av

den lägsta rekommenderade brukskonsentrationen urskiljde väl de olika desinfektionmedlen under beskrivna testförhållanden.

Yttestet gav information om hur desinfektionsmedlet verkade på förorenade ytor. Yttestet är därför ett alternativ till och en tilläggsmetod för suspensionstest vid utvärdering av desinfektionsmedels effekt. Det är emellertid nödvändigt att vidare utveckla yttestet, och så att dess precision fås nöjaktig.

Rengöring av ytor i öppna system

Målet med rengöringsförsöket i öppna system var att utveckla metoder för påvisning av biofilmrester och bakterieceller som blivit kvar på ytorna efter rengöring och desinfektion. Konventionell odling kombinerad med impedansmätning, bildanalys och infärgning med CTC-DAPI gav resultat som var jämförbara och som komplementerade varandra. Resultaten från de olika mätmetoderna möjliggjorde en fullständigare utvärdering av avlägsning och avdödning av bakterieceller än en utvärdering baserad på endast en metod. Resultaten visade att lågtryckssystemet var effektivt vid avlägsnande av biofilm endast i det fall att skummet i sig själv var effektivt. Skummets effekt berodde på dess förmåga att avlägsna biofilm från arbetsytor i kombination med avdödning av bakterier i biofilm.

Resultaten från experimenten med CTC-DAPI infärgning visade att effekten för de fyra rengöringsprocedurerna var likartade. Behandling med Topax 12 tillsammans med Oxonia Aktiv eller Virkon S var aningen effektivare än behandling med enbart Topax 12. Resultaten tyder dock på att disinfektionsmedlet inverkar ytterst lite på rengöringseffekten. Odlingsresultaten visade större skillnader mellan rengöringsprocedurerna, vilket kan förklaras med stress förorsakad av rengörings- och desinfektionsmedel.

Rengöring av ytor i slutna system

Målet med försöken i slutna system var att utvärdera olika CIP-procedurer och att utveckla svabbningen för påvisning av små mängder biofilm och bakterieceller på ytor efter CIP-behandlingar. Konventionell odling kombinerad med impedansmätning, bildanalys och infärgning med CTC-DAPI gav resultat, som var jämförbara och som komplementerade varandra. Brukskoncentrationerna av ämnena förstörde inte alla bakterieceller som växte i biofilmen på ytan.

Rengöringseffekten var bättre på smutsfria än på nedsmutsade ytor. Ingen av behandlingarna avlägsnade all biofilm och ett litet antal levande bakterier stannade kvar på plattorna. Endosporer av *Bacillus thuringiensis* var bättre fästa vid ytorna än biofilmen. Endosporerna innehar också en hög resistens mot värme och kemikalier. De erhållna resultaten påvisade intressanta fakta om de metoder som användes vid hygientestningen, exempelvis kan slembildning på nedsmutsade ytor leda till hög täckningsgrad även i sådana fall då antalet celler vid odling var relativt lågt.

Rengöringsmedlets effektivitet grundar sig på dess förmåga att avlägsna biofilm från ytor i kombination med dess förmåga att avdöda bakterieceller i biofilmen. Kelaterande ämnen i rengöringslösningar verkade förbättra avlägsnandet av biofilm från processytor, ofta fanns det dock levande bakterier på ytorna. Behandling med natriumhydroxid innehållande StabiCIP EA och MIP SP var effektivare vid avlägsning av biofilm än enbart natriumhydroxid och natriumhydroxid innehållande SU560 (resultaten förevisas inte). Användning av olika testmetoder och testmikrober vid utvärdering av rengöringsprocedurer i pilotskala möjliggör tolkningar av rengörings- och desinfektionsresultat uppnådda i prosesskala.

Testing av metabolska indikatorer

Resultaten visade att LIVE/DEAD infärgingen är en snabb, enkel och pålitlig metod för undersökning av suspenderade och svabbade biofilmbakterier. Samverkan mellan polysackarider i biofilm matrix och färgmedlet medför att metoden inte kan användas för undersökning av mikrober fästade på ytor.

Kombinationen CTC-DAPI är också en pålitlig metod för undersökning av celler i suspension och av biofilm bacteria. Dessutom kan man använda en modifierad version av infärgningen för mätning av biofilmbakterier direkt på ytan. Fixering av CTC med formalin möjliggjorde bildanalys av biofilmbakterier fästa på ytor. Inkuberingsperioden var 2,5 h. Tiden som krävs för färgning med LIVE/DEAD var 15 min. CTC-DAPI resultaten erhållna från suspensioner och svabbade biofilmbakterier var lika tillförlitliga som resultaten erhållna med LIVE/DEAD färgning.

Enligt resultaten var mätning av ATP inte tillförlitlig i experimenten under givna förhållanden. Antalet kolonier i en två dagar gammal biofilm var inte tillräckligt stort, för att stabila värden skulle uppnås. ATP-mätning av bakteriemängden i suspensioner var mera tillförlitligt än för biofilmbakterierna.

Testing av nya lösningar för svabbning

Jämförelse av odlings- och mikroskopiresultaten visade att provtagningen från ytor förbättrades då svabbarna fuktats med lösning innehållande ytaktiva ämnen eller enzym tillsammans med kemiska slipmedel. Odlingsresultaten visade att provtaging, där dessa syntetiska lösningar användes, var effektivare än traditionell svabbning. Detta trots att CIP-behandlingen vanligen gör cellerna känsliga för kemikalier. Mikroskopering och mätning av impedans av svabbade ytor visade att en del av biofilmen fanns kvar på ytorna efter behandlingen. Syntetiska lösningar för provtagning bör utvärderas från gång till gång så att man tar hänsyn till processförhållanden, tillväxtförhållanden för biofilmen och önskad känslighet vid provtagningen.

Testing av desinfektion i industriell skala

Kontrollerade forsök med dimning eller desinfektion i industriell skala utfördes vid två ostproducerande mejerier. Ur luftproven analyserades totala antalet mögel och bakterier. Ur ytproven analyserades antalet mögel, koliformer och total antalet bakterier, i vilket mjölksyrabakterierna inte medräknats. Provtagningen utfördes både före produktionsstart och under produktionen. Undersökningarna i båda mejerierna visade klart att en noggrann kontroll och uppföljning av dimnings-systemet behövs. Kritiska punkter som bör kontrolleras var kvaliteten på och mängden av dimma samt konsentrationen för desinfektionsmedlet och sköljnings-processen. Funktionsfel kan bero på t.ex. variation i vattentrycket, felaktig montering och justering av systemet samt täppning av filter. Inget av försöken visade klar positiv effekt av dimningen enligt de använda mikrobiologiska parametrarna. I det fall att de synliga negativa effekterna var verkliga och mikrobtalet ökade till följd av dimningen, kan detta förklaras med funktionsfel i systemet, ökad luftfuktighet eller för låg koncentration av desinfektionsmedel. En annan möjlig förklaring kan vara att resultaten var tillfälliga, de naturliga variationerna för stora och analyserna för få för att man skall kunna göra en bedömning av den verkliga effekten.

Effekten av sköljningsperioden i experimenten var ifrågasatt. Den visuella övervakning gav intrycket av att vattenmängden var otillräcklig för att man skulle kunna avlägsna desinfektionsmedel från horisontala ytor. Otillräcklig sköljning är emot allmänna regler. Å andra sidan förlängs perioden vilket leder till en förhöjd avdödningseffekt med en påföljande förbättring av desinfektionseffekten. I experimenten var alla valda kontrollpunkter kritiska punkter för upprätthållande av god hygien. Många av dem iaktogs speciellt vid standard rengöringen, vilket också innebar extra desinfektion. Trots detta visade analysresultaten att värdena för dessa punkter varierade från ”noll” en dag till ”överväxt” en annan dag. Det är klart att sådana variationer inte inträffar som en effekt av dimningen. Variationerna vilka beskrevs ovan representerar de vanligaste problemen vid testning i industriell skala. Utmaningen är att välja de bästa och mest representativa punkterna vid testning, att välja rätta parametrar för analysering och att hålla oväntade variationer så små som möjligt. Det skulle vara nödvändigt att göra testkörningar som är längre än den period som fanns till förfogande i projektet. Svårigheterna med att undersöka dimning i industriell skala visar klart behovet av neutrala och kontrollerbara test i pilotskala.

RAPPORT ”SANITERING I MEJERI”
ABSTRACT

Prosjektet ble innledet med en undersøkelse av hvilke kommersielle vaske- og desinfeksjonsmidler som er i bruk i nordiske næringsmiddelbedrifter. Utfra dette ble det valgt et antall av de hyppigst brukte midlene for videre studier. Ulike testmetoder og uoverensstemmelser mellom de vitenskapelige resultatene som hittil er oppnådd, understreket viktigheten av å utvikle standard prosedyrer for testing av midler for bruk i meieribedrifter. Parametere anvendt i skumvasking i åpne systemer og prosedyrer for lukket systemer (CIP) dannet grunnlag for utvikling av saniterings- eksperimenter i pilot skala. Testing av desinfeksjonsmidler ble utført på mikroorganismer både i suspensjon og tørket på overflater.

Tradisjonell platespredning hvor antall levende celler som er i stand til å danne kolonier på agar enumeres, ble valgt som rutinemetode. En teknikk for farging av celler ble utviklet, og svaberteknikken med bruk av nye prøvetakingsmidler ble vurdert i pilot- og prosesskala. Sammenlikning av resultatene fra platespredning og mikroskopering viste at prøvetaking fra en overflate var bedret når svabrene ble fuktet med surfaktanter eller enzymer og slipemidler. Biofilmrester og smuss som ble igjen på overflatene, kunne undersøkes ved billedanalyse av preparater farget med acridine orange. Mikroskopiteknikk hvor en brukte DNA farging og metabolske indikatorer, gjorde det mulig å differensiere mellom de levende og døde cellene som ble igjen på overflatene etter behandling. I tillegg ble måling av impedans, bruk av kontaktagar, agar støping og ATP brukt i undersøkelsene.

Seks **desinfeksjonsmidler** ble valgt av meierisamarbeidspartnerne Valio Ltd. og Norske Meierier, og **tested** som anbefalt av CEN (prEN 1040 og 1276). Standard test-mikroorganismene ble tested i tillegg til bedervelses- og sykdomsfremkallende bakterier og bakteriesporer (endosporer) som forårsaker problemer i meieri-industrien. Det ble ikke funnet noen forskjell i de ulike desinfeksjonsmidlene når de ble testet ved prEN 1040 metoden, men høye nivåer av forstyrrende substans, f eks skummet melk og hardt vann brukt i prEN 1276, reduserte virkningen av desinfeksjonsmidlene i varierende grad. *Staphylococcus aureus* og *Pseudomonas aeruginosa* var de mest resistente mot samtlige desinfeksjonsmidler. Blant de fire ”industrielle stammene” var *Salmonella infantis* vanskeligst å drepe. Sporer av *Bacillus cereus* og *Bacillus thuringiensis* ble redusert med mindre enn 1 log enhet i suspensjonstesten. Bruk av 80% av den lavest anbefalte brukerkonsentrasjonen viste å skille godt effekten av de ulike desinfeksjonsmidlene under de beskrevne testforholdene i prEN 1276 hvor den alkoholbaserte IPA 300 var den mest effektiv av de undersøkte midlene. Det bør innføres enda strengere kriterier i testene dersom en vil undersøke virkningen av desinfeksjonsmidler under de aktuelle praktiske forhold. Resultater ved bruk av en kvantitativ overflatetest (CEN TC 216) viste at *Pseudomonas fragi* tørket på overflater var mer resistent mot desinfeksjonsmidlene enn celler i suspensjon.

Eksperimentene med rengjøring av lukkede systemer viste at behandling med alkaliske rengjøringsagenter som inneholdt et chelerende middel i kombinasjon med kalium hydroksid, fjernet smuss og biofilm mer effektivt enn et alkalisk middel med kun natrium hydroksid. Egnetheten av rengjøringsagenten var avhengig av smusskonsistensen, type bakterier på overflaten og overflaten. Resultatene fra mikroskopiske undersøkelser og impedansmålinger etter svabring av overflatene viste at rester av biofilm ble igjen etter samtlige rengjørings-prosedyrer.

Resultatene fra **testene med lavtrykks skumrengjøring** av åpne systemer viste at behandling med en sterk alkalisk rengjøringsagent som inneholdt hypoklorit, fjernet biofilm mer effektivt enn en mildere alkalisk agent anbefalt for overflater utenom prosess. Bakterieantallet på overflatene uten smuss var lavere enn på de tilsmussete overflatene. Resultatene tyder på at lavtrykkssystemet er avhengig av bruk av et meget effektivt skumrengjøringsmiddel hvis all biofilm skal fjernes fra overflatene. Skumrengjøringsmidlet må være i kontakt med overflaten i tilstrekkelig lang tid uten tørking. Effektiviteten av skumrengjøringsagenten er avhengig av at den både fjerner biofilmlagene fra arbeidsoverflatene og dreper alle bakteriene i biofilmen.

Målet med testing av **”fogging”-(tåkelegging-)desinfeksjon i industriell skala** var å undersøke effekten av behandlingen på eksponerte overflater på ulike steder i rommet. Fogging kan defineres som ”kjemisk desinfeksjon ved automatisk dusjing med desinfeksjonsmidlet i et lukket rom”. Kontrollerte forsøk med fogging ble utført i to osteproduserende meierier. Ingen av forsøkene viste noen tydelig reduksjon i den mikrobielle belastningen. Eksperimentene ved begge meieriene påviste klart nødvendigheten av grundig kontroll og oppfølging av prosedyren. Kritiske punkter var kvaliteten på og mengde tåke, konsentrasjon av desinfeksjonsmidlet og skyllingsprosessen. Utilstrekkelig skylling fortynnet bare desinfeksjonsmidlet og rester ble liggende igjen. Tåkelegging øket også luftfuktigheten i rommet, noe som kan bidra til økt korrosjon og ødeleggelse av elektrisk utstyr. Konklusjonene var at effekten av tåkelegging var tvilsom, særlig mhp kritiske punkter og i tørre rom.

KONKLUSJONER I RAPPORTEN

Testing av desinfeksjonsmidler på bakterier i suspensjon og tørket på overflater

Suspensjonstesten beskrevet i prEN 1276 gir opplysninger om hvordan effekten av et desinfeksjonsmiddel er och hur middlet påvirkes av protein og hardt vann. I meierier får bakterier muligens økt resistens som følge av at de lett fester seg til og vokser på de våte overflatene. Det er derfor viktig at suspensjonstestene etterfølges av praktiske vurderinger vha tester i simulert meierimiljø.

Blant standardstammene var *Pseudomonas aeruginosa* vanskeligst å drepe. Av de fire teststammene som representerte problembakterier i meieriindustrien, var *Salmonella infantis* vanskeligst å drepe. Endosporer var også resistente mot desinfeksjonsmidler. Det må ikke forventes at den anbefalte brukskonsentrasjonen som er ment å drepe vegetative bakterier, skal kunne drepe endosporer. Bruk av 80 % av den lavest anbefalte brukerkonsentrasjonen viste å skille godt effekten av de ulike desinfeksjonsmidlene under de beskrevne testforholdene.

Overflatetesten er sannsynligvis strengere enn suspensjonstesten og gir opplysninger om hvordan desinfeksjonsmidler virker på forurensede overflater. Overflatetesten er derfor et alternativ og en tilleggsmetode til suspensjonstesten for evaluering av effektiviteten av desinfeksjonsmidler, men det er nødvendig å arbeide videre med denne testen for å utprøve metodens nøyaktighet.

Rengjøring av overflater i åpne systemer

Målet med forsøket med rengjøring av åpne systemer var å utvikle metoder for påvisning av biofilmrester og bakterieceller som blir igjen på overflatene etter rengjøring og desinfeksjon. Platespredning og impedansmålinger sammen med billedanalyse og CTC-DAPI farging ga resultater som var sammenlignbare, som utfylte hverandre og muliggjorde en totalvurdering av både fjerning av biofilm og drap av bakterieceller.

Effektiviteten av skumrengjøringsagenten er avhengig av at den både fjerner biofilmlagene fra arbeidsoverflatene og dreper alle bakteriene i biofilmen. Resultatene tydet på at lavtrykkssystemet var avhengig av bruk av et meget effektivt skumrengjøringsmiddel hvis all biofilm skulle fjernes fra overflatene. Resultatene viste også at desinfisering etter applikasjon av mildt skum-rengjøringsmiddel har overraskende liten effekt. Skumrengjøringsmidlet må være i kontakt med overflaten i tilstrekkelig lang tid uten tørking.

Rengjøring av overflater i lukkede systemer

Målet med forsøkene var å evaluere ulike CIP-prosedyrer og å utvikle svabermetoder for påvisning av gjenværende biofilm og bakterieceller etter CIP behandlinger. Rengjøringseffekten var bedre på smussfrie enn på tilsmussede overflater. Ingen av behandlingene fjernet all biofilm og et lavt antall levende bakterier ble funnet igjen på kupongene. Endosporene til *Bacillus thuringiensis* festet seg bedre når de ble sprøytet direkte på overflatene enn når de var tilstede i biofilm. Endosporedannende matbårne bakterier har høy resistens mot varme og kjemikalier. Det ble observert at slimdannelse på tilsmussete overflater kan føre til stor arealdekning selv i tilfeller hvor celleantallet er relativt lavt. Resultatene gir interessante opplysninger mhp metodene brukt i hygienetesting.

Effektiviteten av rengjøringsmidlet vurderes på grunnlag av dets evne til å fjerne biofilm fra arbeidsoverflaten samtidig med drap av bakteriene som er tilstede i biofilmen. Chelerende agenter i vaskemidlet så ut til å øke fjerning av biofilm, men i mange tilfeller ble levende bakterier funnet igjen på overflatene etter

behandlingen. Behandlinger med natriumhydroksid som inneholdt StabiCIP EA og MIP SP, fjernet biofilm mer effektivt enn natriumhydroksid alene eller natriumhydroksid med SU560 (resultatene er ikke presentert). Bruk av ulike testmetoder og testmikroorganismer i evaluering av rengjøringsprosedyrer i pilotskala muligjør tolkning av resultatene oppnådde i prosesskala med kommersielle rengjørings- og desinfekjonsmidler.

Testing av metabolske indikatorer

Resultatene viste at LIVE/DEAD fargingsprosedyren er en rask, lettvint og pålitelig metode for undersøkelse av suspenderte og svabrete biofilmbakterier. Pga interaksjoner mellom polysakkarider i biofilm matrix og fargestoffet er metoden imidlertid ikke egnet til undersøkelse av biofilm festet på overflater.

Kombinasjonen CTC-DAPI er også en velegnet fargemetode til undersøkelse av suspenderte og svabrete biofilmbakterier. I tillegg kan en modifisert CTC-DAPI fargeprosedyre brukes til å farge biofilm direkte på overflater. Tilsetting av formalin til CTC muliggjorde billedanalyse av biofilmbakterier festet til overflatene. ATP målingene var ikke følsomme nok til å oppnå pålitelig vurderinger av biofilm eller svabrete biofilmbakterier.

Testing av nye løsninger for svabring

Sammenligning av resultatene fra platespredningene og fra mikroskopi-undersøkelsene viste at prøvetaking fra overflatene ble forbedret ved bruk av svabere fuktet med løsning av syntetisk vaskemiddel som inneholdt surfaktanter eller enzymer sammen med kjemiske slipemidler. Prøvetaking ved bruk av disse nye syntetiske løsninger viste seg å være mer effektiv enn den tradisjonelle metoden, til tross for at skadene pga CIP-behandlingen gjør cellene mer sensitive overfor de toksiske påvirkningene av løsningene. Mikroskopiundersøkelsene og impedansmålingene av overflatene etter svabring påviste noen biofilmrester på overflatene. Syntetiske løsninger egnet for prøvetaking bør vurderes fra gang til gang hvor en tar hensyn til prosess, vekstforholdene for biofilm og den ønskete følsomheten på prøvetakingen.

Testing av desinfeksjon i industriell skala

Kontrollerte forsøk med tåkelegging i industriell skala er utført ved to osteproduserende meierier. Prøver fra luft var analysert for totalantall mugg og bakterier. Overflateprøvene ble analysert for antall mugg, koliforme bakterier og total antall bakterier. Det ble tatt prøver både før produksjonsstart og under selve produksjonen. Studiene ved begge meieriene viste tydelig at det er behov for grundig kontroll og oppfølging av tåkeleggingssystemer. Kritiske punkter er kvaliteten og mengde tåke, konsentrasjon av desinfeksjonsmidlet og effektiviteten av skylling. Funksjonsfeil kan skylles variasjoner i vanntrykket, feilmontering og justering av systemet, tette filtre o.l. Ingen av forsøkene viste klar positiv effekt av tåkeleggingen på de utvalgte mikrobiologiske parameterene. Hvis de

tilsynelatende negative effektene er korrekte og mikroorganismene har øket i antall som følge av tåkeleggingen, kan dette forklares ved funksjonsfeil, økt luftfuktighet og for lave konsentrasjoner av desinfeksjonsmidlet. En annen mulig forklaring er at resultatene er tilfeldige, de naturlige variasjonene for store og analysene for få til å kunne bedømme effektiviteten.

Det stilles spørsmål om effektiviteten av skylleprosedyren. Visuell overvåking gav inntrykket av at vannmengden var utilstrekkelig til å kunne fjerne desinfeksjons-midlet fra vannrette overflater. Det er derfor nødvendig å videreutvikle prosedyrene for prøvetaking og selektere riktige parametere for analyse for å holde alle uønskede variasjoner så lave som mulige. Det ville være nødvendig å foreta testing over en lengre periode enn tilfellet var i dette prosjektet, og det ville være en stor fordel å kunne utføre kontrollerte undersøkelser i pilotskala.

RAPORTTI ”SANITERING I MEJERI” TIIVISTELMÄ

Projektin alussa tehtiin katsaus Pohjoismaisessa meijeriteollisuudessa käytetyistä kaupallisista pesu- ja desinfiointiaineista. Tämän katsauksen perusteella valittiin erityyppisiä paljon käytössä olevia pesu- ja desinfiointiaineita. Näiden aineiden testausmenetelmien ja tieteellisten tulosten vaihtelu motivoi harmonisoidun testausjärjestelmän valmistamisen meijeriteollisuudessa käytetyille aineille. Teollisuuden avoimissa vaahtopesuissa ja suljetuissa CIP-proseduureissa käytettäviä parametrejä käytettiin pilot-mittakaavan puhdistuskokeiden suunnittelussa. Kokeiluihin sisällytettiin desinfiointitestit sekä suspensiolle että pinnoille.

Perinteinen viljely valittiin rutiinimenetelmäksi. Viljelymenetelmä arvioi elävien, agarilla lisääntymiskykyisten, bakteerien lukumäärän. Projektissa kehitettiin värjäysmenetelmä ja arvioitiin pilot- ja prosessimittakaavassa tupostustekniikka, jossa käytettiin uusia näytteenottoliuoksia tupotuksen tehon parantamiseksi. Viljelytulokset, samoin kuin mikroskopointitulokset osoittivat, että näytteenotto pinnasta parantui kun tupot kostutettiin aineella, joka sisälsi pinta-aktiivisia aineita tai entsyymejä ja hankaavaa ainetta. DNA ja metabolisia värejä käyttävä mikroskooppitekniikka mahdollisti pinnalle jääneiden elävien ja kuolleiden solujen erottamisen. Lisäksi määrityksissä käytettiin menetelmiä kuten impedanssi, kontakti agarit, agarvalu ja ATP.

Meijeriyritysten, Valio Oy:n ja Norjan Meijerien, valitsemien kuuden **desinfiointiaineen testaus** toteutettiin käyttämällä CEN:n (prEN 1040 ja 1276) suosittelemia testejä. Käytetyt mikrobit olivat standardi testiorganismeja kuten myös meijerien kannalta huolestuttavia patogeeneja, pilaantumista aiheuttavia bakteereja ja itiöitä. prEN 1040 ei erottanut kuutta desinfiointiainetta toisistaan. Suuret pitoisuudet häiritseviä ainesosia, esim. rasvaton maito ja kova vesi, joita käytettiin prEN 1276:ssa, vähensivät desinfiointiaineiden tehoa huomattavasti. *Staphylococcus aureus* ja *Pseudomonas aeruginosa* olivat vastustuskykyisempiä testatuille desinfiointiaineille. Neljästä teollisuuskannasta *Salmonella infantis* oli kaikkein hankalin tappaa. Suspensiotestissa *Bacillus cereus*- ja *Bacillus thuringiensis*-bakteerien itiöt vähenivät vähemmän kuin 1 log-yksikön. Käytettäessä 80% alhaisimmasta suositellusta konsentraatiosta prEN 1276 -testissä saatiin selviä eroavuuksia desinfiointiaineiden tehokkuuksien välille esitetyissä testiolosuhteissa. Tulokset osoittivat, että alkoholipohjainen IPA 300 oli tehokkain testatuista aineista. Tulokset osoittivat myös, että pinnoille kuivattu *Pseudomonas fragi* oli desinfiointiaineille vastustuskykyisempi kuin suspensiossa olevat solut.

Suljettujen järjestelmien puhdistuskokeissa saadut tulokset osoittivat, että käsittely kelatoivalla aineella sisältävällä emäksisellä pesuaineella yhdistettynä kaliumhydroksidiin poisti likaa ja biofilmiä tehokkaammin kuin emäksinen aine, joka sisälsi ainoastaan natriumhydroksidia. Pesuaineen soveltuvuus riippui lian

koostumuksesta, pinnalta poistettavien bakteerien tyypistä ja pintamateriaalista. Pintojen tupotuksen jälkeen saadut mikroskooppisten ja impedanssimääritysten tulokset osoittivat, että pinnoille jäi jonkinverran biofilmiä kaikkien pesutoimenpiteiden jälkeen.

Avoimien järjestelmien puhdistuskokeissa saadut tulokset osoittivat, että käsittely väkevällä pesuaineella, joka sisälsi hypokloriittia poisti biofilmiä tehokkaammin kuin heikompi emäksinen aine, joka oli suunniteltu muille kuin prosessipinnoille. Bakteerien lukumäärä puhtailla pinnoilla oli alhaisempi kuin esiliatuilla pinnoilla. Tulosten mukaan pesu matalapaineella ei kuitenkaan poistanut kaikkea biofilmiä ellei vaahdotusaine itsessään ollut erittäin tehokas. Tämän vuoksi vaahdotusaineen täytyy pysyä kuivumatta pinnalla tarpeeksi pitkään. Vaahdotusaineen teho riippui sen kyvystä sekä poistaa pintaan kiinnittynyt biofilmi että tappaa bakteerit biofilmin sisällä.

Sumutus tai teollisuusmittakaavan desinfiointi testauksen tarkoitus oli tutkia altistettujen pintojen desinfioinnin tehoa huoneen eri paikoissa. Sumutus voidaan määritellä: “desinfiointiaineen automaattisen suihkutuksen aiheuttama suljetun huoneen kemiallinen desinfiointi”. Kontrolloidut sumutuskokeilut tehtiin kahdessa juustoja tuottavassa meijerissä. Kumpikaan sumutuskokeiluista ei osoittanut mikrobiologisen kuormituksen vähenemistä. Molempien meijerien kokeilut osoittivat selvästi tarpeen perusteelliselle kontrollille ja seurannalle sumutusta suorittaessa. Kriittiset pisteet olivat laatu ja sumun määrä, desinfiointiaineen konsentraatio ja huuhtelu. Riittämätön huuhtelu ainoastaan laimensi desinfiointiainetta. Lisäksi desinfiointiaine ei poistunut kokonaan. Sumutus myös lisäsi huoneen kosteutta, joka saattaa lisätä korroosiota ja vahingoittaa sähkölaitteita. Sumutuskokeilun johtopäätöksenä voidaan todeta, että teho oli kyseenalainen etenkin kriittisissä pisteissä ja myöskin kuivissa huoneissa.

YHTEENVETO RAPORTISTA

Desinfiointiaineiden testaaminen suspensiossa ja pinnoilla

Suspensiotestillä (menetelmäohjeet prEN 1276:ssa) voidaan tutkia desinfiointi-ainetta ja kuinka proteiinit sekä veden kovuus vaikuttavat sen tehokkuuteen. Meijereissä bakteerit saattavat kestää pesutoimenpiteitä paremmin, koska kiinnittymisen jälkeen ne ovat kasvaneet märällä pinnalla. Tämän vuoksi suspensiotestien lisäksi tulisi tehdä käytännön arviointitesti, joka jäljittelee meijeriympäristöä.

Standardikannoista hankalimmin tuhoutuva oli *Pseudomonas aeruginosa*. Neljästä meijeriteollisuuden haittamikrobeja edustavasta testikannasta *Salmonella infantis* oli hankalimmin tuhoutuva. Itiölliset solut olivat myös vastustuskykyisiä desinfiointiaineille, eikä itiöllisten solujen voida olettaa tuhoutuvan kun käytetään vegetatiivisten bakteerien tuhoamiseen tarkoitettuja desinfiointiaineen konsentraatioita. Käytettäessä 80% alhaisimmasta suositellusta konsentraatiosta

prEN 1276 -testissä saatiin selviä eroavuuksia desinfiointiaineiden tehokkuuksien välille esitetyissä testiolosuhteissa.

Pintatesti on luultavasti rajoittavampi (sitovampi, tiukempi) kuin suspensiotesti ja se antaa tietoa siitä miten desinfiointiaineet toimivat kontaminoiduilla pinnoilla. Tästä johtuen pintatesti on vaihtoehto ja lisämenetelmä suspensiotestille arvioitaessa desinfiointiaineiden tehokkuuksia. Menetelmän tarkkuutta on kuitenkin vielä kehitettävä.

Pintatesti on todennäköisesti vaativampi menetelmä suspensiotestiin verrattuna ja sillä voidaan tutkia aineiden tehokkuutta kontaminoituneiden pintojen desinfiointiin. Pintatestiä voidaankin pitää vaihtoehtona ja lisämenetelmänä suspensiotestille arvioitaessa desinfiointiaineiden tehokkuutta. Menetelmän tarkkuutta on kuitenkin vielä kehitettävä.

Pintojen puhdistaminen avoimissa järjestelmissä

Avoimien järjestelmien puhdistusvuuskokeissa oli tarkoitus kehittää mikro-biologisia menetelmiä pintojen puhdistuksen ja desinfioinnin jälkeen pinnalle jääneiden bakteerisolujen ja biofilmin havaitsemiseen. Perinteinen viljely verrattuna sähkönjohtokykymäärityksiin yhdessä kuva-analysoinnin ja CTC-DAPI-värjäyksen kanssa antoivat tuloksia, jotka olivat vertailukelpoisia, toisiaan täydentäviä ja mahdollistivat sekä biofilmin poistamisen että bakteerisolujen tuhoutumisen arvioinnin. Tulokset osoittivat, että matalapainejärjestelmän sovellukset eivät olleet tehokkaita poistamaan kaikkea biofilmiä ellei käytetty vaahtopesuaine itsessään ollut tehokas. Vaahtopesuaineen tehokkuus oli riippuvainen sen kyvystä poistaa biofilmi työpinnalta yhdistettynä sen kykyyn tuhota biofilmissä olevia mikrobeja. Vaahtopesuaineen täytyy tämän vuoksi pysyä kontaktissa pinnan kanssa riittävän kauan kuivumatta.

CTC-DAPI värjäyksellä tehdyt NORDFOOD-testin tulokset osoittivat, että kaikkien neljän puhdistuskäsittelyn vaikutus oli melko samanlainen. Käsittely, jossa käytettiin Topax 12 yhdistettynä Oxonia Aktiviin tai Virkon S:ään, näytti olevan tehokkaampi kuin käsittely pelkällä Topax 12:lla. Nämä tulokset osoittivat, että desinfiointiaineet vaikuttavat yllättävän vähän puhdistuvuuteen. Viljely-menetelmällä saaduilla tuloksilla saatiin suurempia eroavuuksia puhdistus-menetelmien välillä, mikä osittain selittyy puhdistus- ja desinfiointiaineiden aiheuttamasta rasituksesta.

Pintojen puhdistus suljetuissa järjestelmissä

Suljetuilla järjestelmillä tehtyjen kokeiden tarkoituksena oli arvioida erilaisia CIP-käsittelyjä ja kehittää mikrobiologisia tupotusmenetelmiä CIP-käsittelyjen jälkeen pinnoille jääneiden bakteerisolujen ja biofilmin detektointiin. Perinteinen viljely, kuva-analysointi ja sähkönjohtokyky antoivat vertailukelpoisia tuloksia ja täydensivät toisiaan. Käytetyt puhdistusaineiden konsentraatiot eivät tuhonneet kaikkia kasvavia bakteereita pinnoilta olevista biofilmeistä.

Likaamattomien pintojen puhdistuvuus oli parempi kuin liattujen. Mikään käsittelyistä ei poistanut kaikkea biofilmiä ja näytelevyille jäi vähän eläviä bakteereita. *B. thuringiensiksen* itiöt olivat tarttuneet lujemmin pintaan verrattuna biofilmiin. Itiöitä muodostavilla bakteereilla on yleisesti suuri lämmön ja kemikaalien vastustuskyky. Tulokset antavat mielenkiintoista tietoa hygieniatestauksen menetelmistä. Liman muodostuminen liatuille pinnoille voi kuva-analysoinnissa antaa suuria pinta-ala-arvoja, vaikkakin viljelymenetelmän mukaan elävien solujen määrä on vähäinen.

Puhdistusaineiden tehokkuus on riippuvainen sen kyvystä poistaa biofilmi työpinnalta yhdistettynä sen kykyyn tuhota biofilmissä olevia mikrobeja. Puhdistusliuoksessa olevat kelatoivat aineet tuntuvat edesauttavan biofilmin poistoa prosessipinnoilta, mutta jättävät kuitenkin usein muutamia eläviä bakteereita pinnalle. Käsittelyt MIP SP:tä ja StabiCIP EA:ta sisältävällä natriumhydroksidilla olivat tehokkaampia biofilmin poistoon kuin natrium-hydroksidi ja natriumhydroksidi SU560:lla (viimeiksi mainittuja tuloksia ei ole esitetty tässä raportissa). Useiden menetelmien ja mikrobien käyttö pilot-mittakaavassa suoritetuissa pesukokeissa auttaa prosessimittakaavassa saatujen pesu- ja desinfektioaineiden puhdistustehokkuuksien tulkitsemista.

Metabolisten indikaattoreiden testaus

Saatujen tulosten mukaan LIVE/DEAD-värjäys tarjoaa nopean, helpon ja luotettavan menetelmän suspensiobakteerien ja tupotettujen biofilmibakteerien tutkimiseen. Menetelmä ei kuitenkaan sovellu pinnalla olevalle biofilmille, sillä biofilmimatriisin polysakkaridit häiritsevät väriainetta.

CTC-DAPI-menetelmä tarjoaa myös luotettavan menetelmän suspensiobakteerien ja tupotettujen biofilmibakteerien tutkimukseen. Lisäksi muunneltua CTC-DAPI-värjäysmenetelmää voidaan käyttää pinnoilla olevien biofilmien värjäykseen. CTC:n kiinnitys formaliinillä mahdollisti pinnoille kiinnittyneen biofilmin kuva-analyysin. Inkubointiaika CTC-DAPI:lla oli 2,5 h. Tämä inkubointiaika on merkittävästi pidempi kuin LIVE/DEAD-värjäyksen 15 min. Suspensio- ja biofilmibakteerien tulokset olivat yhtä luotettavia kuin LIVE/DEAD-värjäyksellä saavutetut tulokset.

Saatujen tulosten mukaan, ATP-mittaus ei ollut luotettava metabolinen määritys-menetelmä biofilmille eikä tupotetuille biofilmibakteereille. Solujen määrä 2 d biofilmissä ei ollut tarpeeksi suuri, jotta ATP-mittauksella olisi saatu luotettavia tuloksia. Menetelmä sopi paremmin suspensiossa kuin biofilmissä oleville soluille.

Uusien viljelyyn käytettävien tupotusaineiden testaaminen

Viljelytulosten vertaaminen mikroskopointituloksiin osoitti, että näytteenotto pinnoilta tehostui kun käytettiin entsyymejä ja hankaavia kemikaaleja tai pinta-aktiivisia aineita sisältävillä pesuaineseoksilla kostutettuja tuppoja. Siitä huolimatta, että solut vaurioituvat CIP-käsittelyssä ja tulevat siten erittäin alttiiksi pesuaineliuosten toksisille tekijöille, näytteenotto uusia pesuaineseoksia käyttäen

osoittautui perinteistä näytteenottoa tehokkaammaksi. Tupotettujen pintojen mikroskooppiset ja sähkönjohtokykymääritykset osoittivat, että pinnoille jäi vähäisiä biofilmijäämiä. Näytteenottoon soveltuvat pesuaineliuokset tulisi valita tapauskohtaisesti ottaen huomioon prosessiolosuhteet, mikrobien kasvuympäristö ja tarvittavan määrityksen herkkyystaso.

Teollisuusmittakaavassa tehdyn desinfioinnin testaus

Teollisuusmittakaavassa sumutusmenetelmällä kontrolloidusti tehdyt kokeet toteutettiin kahdessa juustoa valmistavassa meijerissä. Ilmanäytteistä analysoitiin bakteerien, homeiden ja hiivojen kokonaismäärät Pintanäytteistä analysoitiin homeet, hiivat ja koliformiset bakteerit sekä bakteerien kokonaismäärä, johon eivät sisältyneet maitohappobakteerit. Näytteenotto tehtiin sekä ennen tuotannon aloitusta että tuotannon aikana. Tutkimukset kummassakin meijerissä osoittivat selvästi sumutusjärjestelmän perusteellisen valvonnan ja seurannan tarpeen. Tarkkailtavat kriittiset kohdat liittyvät tuotetun sumun laatuun ja määrään, desinfiointiaineen pitoisuuteen ja myös huuhtelun vaikutukseen. Toimintahäiriöt saattavat aiheutua veden paineen vaihtelusta, järjestelmän virheellisestä käynnistyksestä ja säädöstä, tukkeutuneista suodattimista jne. Kumpikaan suoritetuista koesarjoista ei osoittanut selvästi valituilla mikrobiologisilla parametreilla tehdyn sumutuksen positiivista vaikutusta. Jos ilmiselvästi negatiiviset vaikutukset ovat tyypillisiä ja mikrobimäärät lisääntyvät sumutettaessa, selityksenä voi olla ongelmat järjestelmän käynnistyksessä, lisääntynyt kosteus ja desinfiointiaineiden liian alhainen pitoisuus. Toinen mahdollinen selitys on että tulokset ovat satunnaisia ja luonnolliset olosuhteiden vaihtelut ovat liian suuria ja analysoinnit liian suppeita, jotta voitaisiin vetää johtopäätoksiä vaikutuksesta.

Huuhtelun vaikutus kokeissa on kyseenalaista. Silmämääräisen tarkastelun mukaan vesimäärä oli riittämätön poistamaan vaakasuorilla pinnoilla ollutta desinfiointiainetta. Riittämätön huuhtelu on vastoin yleisiä säännöksiä. Toisaalta se pidentää desinfioinnin vaikutusaikaa ja siten parantaa desinfioinnin tulosta. Kokeissa valitut valvontapisteet olivat hyvän hygienian kriittisiä pisteitä. Useisiin niistä kiinnitetään erikoishuomiota tavallisissa puhdistuskäsittelyissä, jotka sisältävät myös lisädesinfioinnin. Tästä huolimatta ja sumutuksen johdosta näiden pisteiden analyysitulokset voivat vaihdella yhden päivän tuloksesta ”nolla” seuraavan päivän tulokseen ”runsas kasvu”. On selvää, etteivät tämäntapaiset vaihtelut aiheudu sumutusjärjestelmän vaikutuksesta. Kuvatut vaihtelut ovat pääasiallisin ongelma tehtäessä kokeita teollisuusmittakaavassa. Vaikeutena on valita parhaat ja edustavimmat testipisteet, valita oikeat analyysiparametrit ja pitää epätoivotut vaihtelut mahdollisimman pieninä. Voisi olla tarpeen testata pidempiä aikajaksoja kuin tässä projektissa. Teollisuusmittakaavassa tutkittavaan sumutukseen liittyvät vaikeudet osoittavat puolueettomien ja valvottujen pilot-mittakaavan testien olevan tarpeen.

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