ESPOO 2002 VTT PUBLICATIONS 481

Gun Wirtanen, Solveig Langsrud, Satu Salo,
Ulla Olofson, Harriet Alnås, Monika Neuman,
Jens Petter Homleid & Tiina Mattila-Sandholm

Evaluation of sanitation procedures for use in dairies

VTT PUBLICATIONS 481

EVALUATION OF SANITATION PROCEDURES FOR USE IN DAIRIES

Gun Wirtanen[1], Solveig Langsrud[2], Satu Salo[1], Ulla Olofson[3], Harriet Alnås[4], Monika Neuman[3], Jens Petter Homleid[5] and Tiina Mattila-Sandholm[1]

[1] VTT Biotechnology, Espoo, Finland

[2] Matforsk, Ås, Norway

[3] SIK, Göteborg, Sweden

[4] Arla Foods, Göteborg, Sweden

[5] TINE, Oslo, Norway

ISBN 951–38–6017–5 (soft back ed.)
ISSN 1235–0621 (soft back ed.)

ISBN 951–38–6018–3 (URL: http://www.inf.vtt.fi/pdf/)
ISSN 1455–0849 (URL: http://www.inf.vtt.fi/pdf/)

Copyright © VTT Technical Research Centre of Finland 2002

JULKAISIJA – UTGIVARE – PUBLISHER

VTT, Vuorimiehentie 5, PL 2000, 02044 VTT
puh. vaihde (09) 4561, faksi (09) 456 4374

VTT, Bergsmansvägen 5, PB 2000, 02044 VTT
tel. växel (09) 4561, fax (09) 456 4374

VTT Technical Research Centre of Finland, Vuorimiehentie 5, P.O.Box 2000, FIN–02044 VTT, Finland
phone internat. + 358 9 4561, fax + 358 9 456 4374

VTT Biotekniikka, Tietotie 2, PL 1500, 02044 VTT
puh. vaihde (09) 4561, telekopio (09) 455 2103

VTT Bioteknik, Datavägen 2, PB 1500, 02044 VTT
tel. växel (09) 4561, telefax (09) 455 2103

VTT Biotechnology, Tietotie 2, P.O. Box 1500, FIN-02044 VTT, Finland
phone internat. +358 9 4561, telefax +358 9 455 2103

Cover painted by freelance artist Antti Huovinen

Technical editing Leena Ukskoski

Otamedia Oy, Espoo 2002

Wirtanen, Gun, Langsrud, Solveig, Salo, Satu, Olofson, Ulla, Alnås, Harriet, Neuman, Monika, Homleid, Jens Petter & Mattila-Sandholm, Tiina. Evaluation of sanitation procedures for use in dairies. Espoo 2002. VTT Publications 481. 96 p. + app. 43 p.

Keywords NORDFOOD, dairies, sanitation, hygiene, microbes, detection, isolation, disinfection, cleaning, life-cycle analysis, environmental assessment

ABSTRACT

The research work for project P96049 in the second NORDFOOD programme was carried out at the Nordic research institutes VTT Biotechnology, MATFORSK and SIK as well as at the universities in Helsinki and Reykjavik from April 1997 to January 2000. The companies involved in the project were the dairies Valio Ltd. from Finland, Arla from Sweden and TINE from Norway as well as the technochemical company Suomen Unilever Oy DiverseyLever from Finland. Dr Gun Wirtanen, VTT Biotechnology, coordinated the project. The senior advisors at Nordic Industrial Fund involved in the project were Maija Uusisuo and Oddur Gunnarsson. The experiments were focused on monitoring methods in sanitation of open and closed systems e.g. fogging, ozonation, footbath hygiene, cleaning of cheese moulds and yoghurt pasteurizers, development of testing procedures for measuring disinfectant efficacy, microbial resistance phenomena against disinfectants, life-cycle assessment (LCA) and an evaluation procedure for the functionality of the cleaning procedures. New procedures in hygiene have been implemented in dairies, based on the results. The findings can be summarized as follows:

- The main task of the research conducted in Sweden was to develop and evaluate practical methods for the measure of cleaning and disinfection efficiency. These methods are suitable for equipment surfaces used for production of various dairy products. The analysing methods, washing out and triphenyltetrazolium chloride (TTC) agar, worked well in testing the cleanability of plastic cheese moulds. As part of this study a 5-step method how to evaluate cleaning and disinfection agents was prepared at Arla Foods.

- *Bacillus cereus* is an important target organism when disinfecting equipment for production of consumer milk in Nordic dairies. The total number of remaining *Bacillus* spores on solid surfaces is a useful indicator for measuring cleaning and disinfection efficiency. Improved methods for detection of surface-adhered spores can be based on fluorescent dyes,

indirect immunofluorescence and quartz crystal microbalance (QCM) techniques.

- Less environmentally harmful cleaning procedures based on ozonated water and enzyme-based agents used in cleaning-in-place (CIP) were investigated. Energy can also be saved using lower temperatures. Promising results with 1–3 log-unit spore-reduction (i.e. 90.0–99.9% reduction) was achieved using an ozone (O_3) concentration of 0.1–0.3 ppm.

- Fogging with different agents including ozone is a potentially useful method for disinfection of production and storage rooms. There are, however, difficulties in reaching areas such as the outside of pipelines, rubber hoses and ceilings. In these areas there are probably attached microbes that can adapt to the disinfectant when exposed at sublethal concentrations or survive because they are resistant to the fogging agent. The results showed that ozone is less effective when the microbes are dry. The performance of the fogging unit should be monitored frequently to ensure optimal disinfection.

- According to a questionaire, footbaths were extensively used in Norwegian dairies. The aim was to combine practical information and laboratory research to develop a maintenance guideline for footbaths. A maintenance guideline is clearly needed, because it is evident that survival, growth and spreading of resistant bacteria occur when using footbaths. The Norwegian report initiated an investigation at Arla Foods in which the microbial status of the footbaths was mapped. Microbial contaminants were found in all footbaths tested, even though the sodium hypochlorite concentration in general was correct or above the recommended level. Based on the results achieved, it is recommended that footwear should be changed, when entering a clean area, instead of using footbaths.

- The LCA of various CIP methods, including standard CIP with lye phase, acid phase and thermic disinfection and enzymatic cleaning followed by acid treatment and chemical disinfection, was performed at TINE. The LCA covered all environmental aspects including transportation and effects on the wastewater. In an LCA all the potential environmental effects of each emission were considered according to the worst case scenario. The LCA provides information necessary for choosing the best CIP method, even though widely varying assumptions must be made and limitations

considered. In this study enzyme-based CIP procedures showed the best results, because enzymes are used in very small concentrations and at low temperature.

- The aim of the sanitation study on yoghurt fermentation lines was to isolate interfering spoilage microbes, possibly thermophilic bacteria, from a yoghurt process and to find suitable agents for cleaning the yoghurt-soiled process surfaces. In the pilot-scale cleaning studies, in which various cleaning agent combinations were compared, yoghurt-milk including process isolates were burned on stainless-steel surfaces using steam heating. The results showed that the best cleaning effect for surfaces with the burned milk-soil was achieved with a 2-phase cleaning procedure using chelator-based sodium hydroxide (NaOH).

- The cheese mould hygiene studies were carried out at both pilot and process scale. The structure of the plastic cheese moulds is complex, with long, narrow conical channels. The ultrasound cleaning procedure was shown to be efficient in cleaning the channels of the cheese moulds. The cleanliness of the cheese moulds both after pilot-scale cleaning and during processing was assessed using various methods, of which the dipslide technique proved to be the most practical for detecting microbial contaminants. In industrial scale, pH measurement proved to be a useful indicator for checking, as long as the cleaning procedure was functional. The chemical oxygen demand (COD) as well as ethylenediaminetetraacetic acid (EDTA) measurements were useful in following up the organic load of the cleaning waters.

- In trials to develop new environmentally less harmful cleaning agents, proteinase samples e.g. cryotin from cod, trypsin from Antarctic krill and chymotrypsin from cod, were prepared at the University of Iceland. A microtitration tray procedure with assessment based on fluorometric, colorimetic and turbidometric methods was developed to study the efficacy of these enzymatic cleaning agents on *Bacillus* biofilms. A method based on the fluorogenic redox indicator resazurin was further used in evaluating the enzymatic cleaning procedures on biofilm bacteria of the lactic-acid bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*. The proteinase samples tested removed biofilms when there was no milk residues on the surfaces.

- To evaluate the influence of fluid dynamic shear (0.024–0.53 m/s) of disinfectant solutions on the extent and mode of detachment of *P. aeruginosa* biofilm, a concentric cylinder reactor (CCR) was used. The results showed that the CCR can be used to discriminate between biocidal and cleansing action for different disinfectant types. In testing of disinfectant efficiency, impedance can be used for evaluating surface sterility because very few, well-adapted, fast-growing cells as well as large amounts of chemically treated cells change the conductance or capacitance values in the liquid. With staining procedures, the viability and total amount of cells can be measured on the surface. Cultivation is not a proper method for measuring well-established biofilms on surfaces, because the cells can stick firmly to the surface material, and thus not all cells are measured using swabs.

- 1-*N*-phenyl-naphthylamine (NPN) uptake measured spectrophotometrically can be used to evaluate the effects of cell sensitizers on both Gram-negative and Gram-positive bacteria. In Gram-positive cells the protective outer membrane (OM) is lacking and NPN has much greater free access to the cytoplasmic membrane. The results of the NPN uptake assay for permeabilization of bacteria by chelators and polyethyleneimine (PEI) showed that the sensitivity towards OM-permeabilizing substances varied considerably between bacterial species. In general, citric acid was an effective permeabilizer, whereas sodium citrate was a much weaker agent.

- Parameters in the test based on hydrogels i.e. biofilm constructs of poloxamer were optimized using various disinfectants. The results showed that a 5-h incubation period of 100 μl of inoculated droplets was the best test matrix. Disinfectant efficacy on biofilm bacteria can be optimized using this method. The results showed that Gram-negative bacteria are more resistant to disinfectant treatments than are Gram-positive bacteria. The hydrogen peroxide-based agents proved to be efficient against most microbes tested.

Development of detection and identification methods for assessing microbial contaminants on or in process equipment, raw material, process air, packaging material and final products should be continued. Cooperation in Nordic dairy hygiene is continuing in a network project DairyNET – Hygiene control in dairy environment funded by the Nordic Industrial Fund (P00027).

PREFACE

The research work for project P96049 in the second NORDFOOD programme was carried out at the Nordic research institutes VTT Biotechnology, Matforsk and SIK as well as at the universities in Helsinki and Reykjavik from April 1997 to January 2000. The companies involved in the project were the dairies Valio Ltd. from Finland, Arla from Sweden and TINE from Norway as well as the technochemical company Suomen Unilever Oy DiverseyLever from Finland. The senior advisors at Nordic Industrial Fund involved in the project were Maija Uusisuo and Oddur Gunnarsson. Dr Gun Wirtanen, VTT Biotechnology, coordinated the project and the project board meetings were chaired by Janna Luotola and Irma Klemetti, both from Valio Ltd. The project group and the steering group have met 8 times during the project. The project was carried out according to plans and the tasks to be carried out were chosen at the steering group meetings.

Financial support for the project, which is gratefully acknowledged, was provided by the Nordic Industrial Fund. The senior advisers for this project at the Nordic Industrial Fund were Maija Uusisuo and Oddur Gunnarsson. The representatives at the participating institutions were: Janna Luotola, Irma Klemetti, Matti Koivisto, Jarmo Juurinen and Kai Hotakainen (Valio Ltd. in Helsinki, Lapinlahti and Herajoki), Jens Petter Homleid (TINE in Oslo), Harriet Alnås and Birgitta Axelsson (Arla FoU in Stockholm and Växjö), Urban Wiik and Kai Ahlgren (Suomen Unilever Oy DiverseyLever in Turku and Helsinki), Solveig Langsrud, Gunhild Sundheim and Birgitta Baardsen (Matforsk at Ås), Ulrika Husmark and Ulla Olofson (SIK in Gothenburg), Jon Bragi Bjarnason (University of Iceland in Reykjavik), Terhi Ali-Vehmas (University of Helsinki in Helsinki) as well as Satu Salo, Tiina Mattila-Sandholm and Gun Wirtanen (VTT Biotechnology in Espoo). The valuable comments of the referees Prof. Anna-Maija Sjöberg from University ofHelsinki and Ass. Prof. Alan Friis Biocentrum-DTU are gratefully acknowledged. Our special thanks are due to Antti Huovinen,who has painted the cover picture.

The experiments were focused on monitoring methods in sanitation of open and closed dairy systems e.g. fogging, ozonation, footbath hygiene, cleaning of cheese moulds and yoghurt pasteurizers as well as development of testing procedures for measuring disinfectant efficacy in suspensions and on surfaces using starved cells in suspensions and cells grown in biofilms and in biofilm-constructs on stainless-steel surfaces. Information on the results obtained in this project has been presented to an extended audience as oral presentations and posters at international symposia and in

articles in international journals. The abstract and summary of the results achieved in this project have also been translated into Swedish, Norwegian, Icelandic and Finnish. The aim of the new Nordic network project DairyNET – Hygiene control in dairy environment (P00027), in which there are partners from all Nordic countries, is to continue the Nordic dairy hygiene research carried out in the 2 previous NordFood programmes (1994–2000) through maintaining contacts between industrial personnel and researchers dealing with hygiene questions in the Nordic countries.

CONTENTS

ABSTRACT 3

PREFACE 7

LIST OF APPENDICES 12

VOCABULARY AND ABBREVIATIONS 14

1. INTRODUCTION TO THE PROJECT 17

2. EVALUATING THE EFFICACY OF SANITATION PROCEDURES 23
   - 2.1 Literature review in dairy hygiene 23
     - 2.1.1 Soiling mechanisms 23
     - 2.1.2 Pathogenic microbes in dairy processes 24
     - 2.1.3 Sanitation procedures 26
   - 2.2 Hygiene of footbaths 27
     - 2.2.1 Use and control of footbaths 27
     - 2.2.2 Hygienic conditions of footbaths 30
   - 2.3 Fogging procedures in dairies 31
     - 2.3.1 Control of fogging disinfection 31
     - 2.3.2 Air disinfection 36
   - 2.4 Hygiene in an open dairy process – Cheese production 40
     - 2.4.1 Cheese production 40
     - 2.4.2 Methods in evaluation of cheese mould cleanliness 41
     - 2.4.3 Ultrasound cleaning procedure in cheese production 43
   - 2.5 Hygiene in closed dairy processes – Milk and yoghurt production 43
     - 2.5.1 Yoghurt production 43
     - 2.5.2 Cleaning procedures used for pasteurizers 44
     - 2.5.3 Ozone compared with chemicals in milk CIP cleaning 45
     - 2.5.4 Potentiation of the disinfectant effect with alkali and enzyme wash 48
   - 2.6 Enzymatic cleaning 50

2.7 Testing of disinfectant efficacy 50
2.7.1 Suspension tests 51
2.7.2 Surface test based on biofilms 52
2.7.3 Surface test based on biofilm constructs 55

3. MICROBIAL PHENOMENA 57
3.1 Resistance phenomenon due to use of disinfectants 57
3.1.1 Definition of resistance 57
3.1.2 Resistant strains from dairies 57
3.2 Thermophilic bacteria in yoghurt processing 58
3.3 *Bacillus* spores on process surfaces – Inventory study of total contamination and *B. cereus* along a cream production line 59

4. ENVIRONMENTAL ASSESSMENT 61
4.1 Life-cycle analysis for assessing environmental effects of cleaning 61
4.2 Evaluation procedure for assessing the functionality of sanitation 62

5. DETECTION METHODS USED IN THE STUDIES 65
5.1 Isolation of resistant microbes 65
5.1.1 Commonly used methods for testing resistance phenomena 65
5.1.2 Methods for isolating resistant strains 66
5.2 Disinfectant testing using biofilm constructs 67
5.3 Disinfectant testing using biofilms formed on surfaces 68
5.4 Disinfectant efficacy testing – Cell permeability testing 70
5.5 Control method for testing footbath hygiene 71
5.6 Method for testing cheese mould cleanability 71
5.7 Method for testing air disinfection efficacy 73
5.8 Evaluation of the cleaning and disinfection efficacy of various CIP procedures using spore-soiled surfaces 74
5.9 Testing of cleaning efficacy on surfaces 75
5.9.1 Process surfaces – Staining of *Bacillus* spores 75
5.9.2 Cleaning of a production line 77
5.10 Preparation of enzymes and efficacy testing of enzymatic cleaning 77
5.10.1 Preparation of proteinase 77
5.10.2 Microtitration tray-based methods in efficacy testing 78

6. CONCLUSIONS 80
6.1 Cleaning of closed systems 80
6.2 Comparison of test methods for disinfectant efficiency 80
6.3 Testing of air disinfection in industrial scale 80
6.4 Surface disinfection in industrial scale 81
6.5 Resistance phenomena due to disinfection 81
6.6 Evaluation of the cleaning of cheese moulds 81
6.7 Environmental assessment 82

REFERENCES 83

APPENDICES

LIST OF APPENDICES

Appendix 1. Summary of the activities in the DAIRYNI-project (P96049)

Appendix 2. Poster presentation Highlights from the NordFood2 Project P96049 Evaluation of cleaning agents and disinfectants for use in dairies: Methods and mechanisms (1997–2000) written by Gun Wirtanen and presented at the conference The future for Nordic food innovation in a European context arranged by Nordic Industrial Fund in Stockholm 28–29 January 2002.

Appendix 3. List of publications, oral and poster presentations as well as theses carried out in the project

Appendix 4. Sammandrag av projektet (in Swedish)

Appendix 5. Sammendrag av prosjektet (in Norwegian)

Appendix 6. Tiivistelmä ja yhteenveto projektista (in Finnish)

Appendix 7. Samantekt (Íslenska)

Appendix 8. Abstract Characterisation of *Serratia marcescens* surviving in disinfecting footbaths written by Solveig Langsrud, Trond Møretrø and Gunhild Sundheim

Appendix 9. Poster presentation Ultrasound cleaning in cheese mold hygiene based on the Pro Gradu thesis by Antti Heino and presented at the IAFP Annual meeting in August 2000.

Appendix 10. Abstract of the oral presentation Comparison of ultrasound based cleaning programs for cheesery utensiles written by Wirtanen, G., Salo, S., Heino, A., Hattula, T. & Mattila-Sandholm, T. and published in the proceedings of Fouling, Cleaning and Disinfection in Food Processing (edited by Wilson, D. I., Fryer, P. J. & Hasting, A. P. M.) held at the Jesus College in Cambridge April 2–4, 2002, pp. 165–171.

Appendix 11. Poster presentation Effects of cleaners of biofouled stainless-steel surfaces in yoghurt manufacturing equipment based on the Master thesis by S. Kontulainen and presented at the IAFP Annual meeting, August 2000.

Appendix 12. Abstract of the article Potentiation of the lethal effect of peroxygen on *Bacillus cereus* spores by alkali and enzyme wash written by Langsrud, S., Baardsen, B. & Sundheim, G. and published in International Journal of Food Microbiology, 56 (2000) 81–86.

Appendix 13. Abstract of the article Influence of fluid dynamic forces upon the steady-state population dynamics in microbial biofilm communities written by Willcock, L., Allison, D. G., Holah, J.,

Wirtanen, G. & Gilbert, P. and published in Journal of Industrial Microbiology, 25 (2000) 235–241.

Appendix 14. Poster presentation Disinfectant testing using bacteria grown in poloxamer-hydrogel biofilm-constructs based on Bachelor theses by Päivi Härkönen and Mervi Aalto and presented at the ASM conference Biofilm 2000, July 2000.

Appendix 15. Colour pictures of various staining techniques presented in the Master thesis Detection of *Bacillus cereus* spores on surfaces using DEM, immunofluorescence and QCM-DTM written by Anna Nilsson.

VOCABULARY AND ABBREVIATIONS

A. niger	*Aspergillus niger*; mould used in the air disinfection experiments
AISI	American Iron and Steel Institute
amphoteric tenside	agent in which the surface-active molecule contains both an anionic and a cationic radical, either of which can be activated under different pH conditions or in extreme environments both simultaneously to form a zwitterion
AO	acridine orange (CA index name: N,N,N',N'-tetramethyl-3,6-acridine-diamine monohydrochloride)
ATP	adenosine 5'-triphosphate (CA index name: adenosine 5'-(tetrahydrogen triphosphate))
AU	auramine O (CA index name: 4,4'-carbonimidoylbis[N,N-dimethyl-benzenamine monohydrochloride)
AY	acridine yellow (CA index name: 2,7-dimethyl-3,6-acridine-diamine mono- hydrochloride)
B.	*Bacillus*; Gram-positive, spore-forming rods belonging to the *Bacillus* family (e.g. *B. cereus, B. flavothermus, B. licheniformis* and *B. subtilis*)
Betane	amphoteric tenside
C. albicans	*Candida albicans*, yeast used in the air disinfection experiments
CCR	concentric cylinder reactor used in disinfectant efficacy studies
CFU	colony-forming units
cleanability	ease of removal of soiling components
cleaning	removal of soiling components
COD	chemical oxygen demand
CIP	cleaning-in-place
CTC	5-cyano-2,3-di-p-tolyltetrazolium chloride (5-cyano-2,3-bis(p-methylphenyl)-2H-tetrazolium chloride)
D. anomala	*Dekkera anomala,* yeast used in disinfectant efficacy studies
DAPI	4',6-diaminidino-2-phenylindole (CA index name: 2-[4-(aminoiminomethyl)-phenyl]-1H-indole-6-carboximidamide)
DEM	direct epifluorescence microscopy

detergent	surface-active agent containing tensides
disinfectant	sanitizer; an agent reducing viable microorganisms through destruction or removal and preventing microbial growth on surfaces during the interproduction period in processing
DRBC agar	dichloranrose bengalchloramphenicol agar
E-value	capacitance or relative electrode impedance value of the BacTrac 4100 equipment
E. coli	*Escherichia coli*, bacterium used in disinfectant efficacy studies
EDTA	organic chelating agent; ethylenediaminetetraacetic acid (CA index name: N,N'-1,2-ethanediylbis[N-(carboxy-methyl)-glycine)
EHEDG	European Hygienic Engineering and Design Group
ERB	erythrosine B (CA index name: 3',6'-dihydroxy-2',4',5',7'-tetraiodospiro [isobenzofuran-1(3H),9'-[9H]xanthen]-3-one disodium salt)
ETA	ethylalcohol-based disinfectants
IPA	isopropylalcohol-based disinfectants
HNO_3	nitric acid
KOH	potassium hydroxide
L. inocua	*Listeria inocua,* bacterium used in disinfectant efficacy studies
L. monocytogenes	*Listeria monocytogenes,* bacterium used in disinfectant efficacy studies
LCA	life-cycle assessment
M-value	conductance or relative medium impedance value of the BacTrac 4100 equipment
M. luteus	*Micrococcus luteus,* bacterium used in disinfectant efficacy studies
MIC	minimum inhibitory concentration
MRD	maximal recovery diluent
NaOH	sodium hydroxide
NB	nutrient broth
NPN	1-*N*-phenyl-naphthylamine
O_3	ozone
OM	outer membrane
ORP	oxidation-reduction potential (value in mS/V)

Oxonia Aktiv	disinfectant containing hydrogen peroxide and peracetic acid
P.	*Pseudomonas;* Gram-negative rods belonging to the genus *Pseudomonas*
PCA	plate count agar
PEI	polyethyleneimine
Poloxamer F127	a diblock copolymer of polyoxyethylene and polyoxypropylene
S.	*Staphylococcus* Gram-positive cocci belonging to the genus *Staphylococcus*
SU 560	chelating additive containing EDTA
surfactant	surface-active agent
TAAS	tenside-based disinfectant
TEGO	disinfectant containing tetraethyleneglycol orthophtalate
tenside	tensio-active substance
TGE agar	tryptone glucose extract agar
TP-99	disinfectant containing alkylaminoacetate
TSA	tryptone soy agar
TSB	tryptone soy broth
TTC	triphenyltetrazolium chloride
UHT	ultrahigh-temperature treated
UV	ultraviolet
QCM	quartz crystal microbalance
Veterinær Ultra Des	disinfectant containing quaternary ammonium compound

1. INTRODUCTION TO THE PROJECT

Cleanliness of surfaces, training of personnel, as well as good manufacturing and design practices are important in combating hygiene problems in the food industry (Holah & Timperley, 1999). Achieving a clean food plant must be desired by the plant management, which must invest the necessary time and money to accomplish it. Careful thought must be given to the cleaning procedures, including the programme, cleaning agents, disinfectants and cleaning equipment used. The key to effective cleaning of a food plant lies in understanding the type and nature of the soiling (e.g. sugar, fat, protein and mineral salts) and of the removal of microbial growth from surfaces. Fats are easily removed at temperatures slightly above their melting point. Sugars and other carbohydrates are water-soluble at elevated temperatures, but temperatures causing caramelization should be avoided. Proteins are denatured at elevated temperatures and may adhere strongly to surfaces at high temperatures. Cleaning and disinfection procedures can be optimized with pilot-scale equipment for both closed and open processes. An efficient cleaning procedure consists of a sequence of rinse, detergent and disinfectant applications at suitable temperatures using efficient concentrations and a final drying phase (Wirtanen & Mattila-Sandholm, 2002).

Microbes commonly found on food contact surfaces include enterobacteria, lactic acid bacteria, micrococci, streptococci, pseudomonads and bacilli (Wirtanen *et al*., 2000). Microbes growing on surfaces have a tendency to form protective extracellular matrices, which are called biofilms. In addition to a surface the microbes need only water to start up this formation. It has been shown that the bacterial slime of *Bacillus* sp. improved the heat resistance of these bacteria, extending the autoclaving time required for successful sterilization to several hours. Biofilm components can also alter the resistance to steam (Mattila-Sandholm & Wirtanen, 1992). The elimination of biofilm is a very difficult and demanding task because many factors such as temperature, time, mechanical and chemical forces affect detachment (Wirtanen, 1995). Disinfection after removal of biofilms, using suitable cleaning procedures, is also required in food plants where wet surfaces provide favourable conditions for the growth of microbes (Exner *et al*., 1987; Mosteller & Bishop, 1993).

In the food industry, equipment design and choice of surface materials (Fig. 1) are crucial to combating biofilm formation (Holah & Timperley, 1999; Wirtanen

& Mattila-Sandholm, 2002). The most useful material component in processing equipment is steel, which can be treated, e.g. with mechanical grinding, brushing, lapping and electrolytic or mechanical polishing. It was reported that although the grain boundaries of AISI (American Iron and Steel Institute) 316L acid-resistant stainless steel constitute 3–20% of the total surface area, over 90% of the adherent bacteria were found attached to the grain boundaries (Bryers & Weightman, 1995).

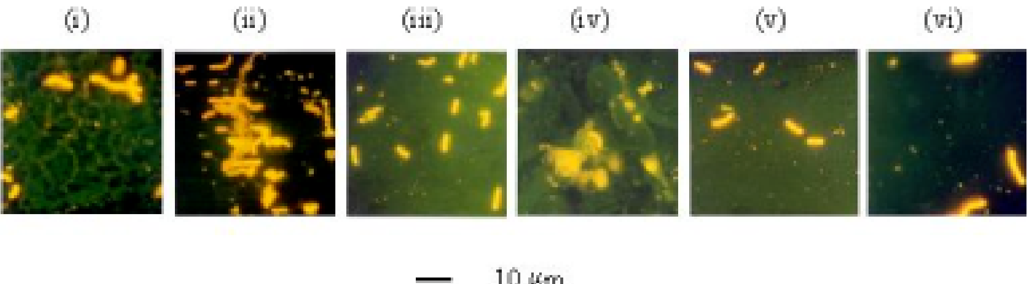


Figure 1. Epifluorescent microscopy photographs of 2-d-old Bacillus subtilis biofilms stained with acridine orange. The biofilms had been grown on various stainless-steel surfaces (AISI 304): (i) standard 2B, (ii) standard 4N, (iii) standard BA, (iv) glass-blasted 2B, (v) brushed 2B and (vi) electrochemically polished 2B surfaces. The scale marker is equivalent to 10 μm (Wirtanen & Mattila-Sandholm, 2002).

Dead ends, corners, cracks, crevices, gaskets, valves and joints are vulnerable points for biofilm accumulation (Pirbazari *et al*., 1990; European Hygienic Engineering and Design Group (EHEDG), 1993a, b; Chisti & Moo-Young, 1994). Poorly designed sampling valves can destroy an entire process or give rise to incorrect information due to biofilm effects at measuring points. As a result of the construction, valves are vulnerable to microbial growth and thus constitute a hygienic risk (Chisti & Moo-Young, 1994; EHEDG, 1994). Hoses, tubes, filters etc. containing polyvinylchloride increase the risk of contamination (Price & Ahearn, 1988). Problems with accumulation of particulates and cells will occur whenever cleaning is inappropriate for any reason (Mettler & Carpentier, 1998). Inadequate cleaning and sanitation of surfaces coated with biofilms represents a source of contamination within the process (Wirtanen *et al*., 2000). In practice, a biofilm left on improperly cleaned surfaces is a barrier between microbes and the disinfectants, antibiotics or biocides used against them

(Kinniment & Wimpenny, 1990; Nichols, 1991; Brown & Gilbert, 1993; Wirtanen *et al*., 2000).

In addition to causing problems in cleaning and hygiene (Hood & Zottola, 1995), biofilm can cause energy losses and blockages in condenser tubes, cooling-fill materials, water and wastewater circuits and heat exchangers (Characklis, 1981). Biofilm can also cause health risks due to the release of pathogens in drinking water distribution systems. In food processing, water supply system biofilms cause problems in granular activated carbon columns, reverse osmosis membranes, ion-exhange systems, degasifiers, water storage tanks and microporous membrane filters (Flemming *et al*., 1992; Mucchetti, 1995). Accumulation of mixed-population biofilms containing sulphate-reducing bacteria causes corrosion in industrial water systems. It must be remembered that monitoring practices based on sampling of the liquid phase do not reflect the location or extent of microbes growing in biofilms on surfaces (Cloete *et al*., 1989).

Legislation on food hygiene and the hygienic design of food machinery, together with public awareness of product quality and manufacturers' desires to improve product safety, makes reliable cleanability testing an important issue. In this type of testing it must be possible to assess the relative cleanability of various equipment components to facilitate the design, testing and maintenance of hygienic food-processing equipment. Assessment must be carried out using standardized test procedures on a sound scientific basis (Cnossen & Wirtanen, 2002). The aim of EHEDG, which is an independent consortium of representatives from research institutes, the food industry, equipment manufacturers and government organizations, is to develop hygienic equipment on a scientifically and technologi-cally sound basis. The effects of cleaning procedures used in the food industry can be evaluated using LCA. All environmental aspects including the process and energy consumped in producing the cleaning chemicals, transportation, properties of chemicals before and after cleaning, water amount used, organic and inorganic loads in wastewater and the recipient. The present report deals with detection and elimination of microbes on surfaces in dairy environments. The project plan is given in Fig. 2 and a summary of the activities in Table 1 (Appendix 1). The contact addresses of the members in the research group and the industrial partners are given in Table 1.

The purposes of the Nordic Industrial Fund project P96049 ”Evaluation of cleaning agents and disinfectants for use in dairies: methods and mechanisms” are to develop environmentally less harmful cleaning agents and disinfectants than those currently available on the market and reliable methods with which the mechanisms of these agents can be studied on microbes in suspensions as well as on surfaces in the laboratory, at pilot and process scales (Appendix 2). The

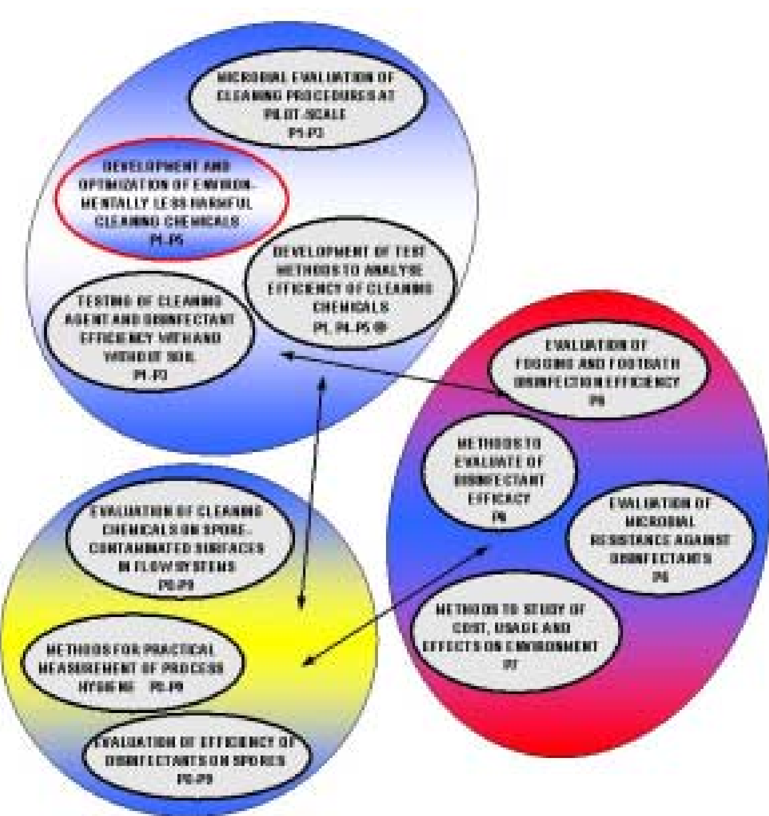


Figure 2. Cooperation between the 3 project groups: P1) VTT [⊗ = cooperation with the universities in Manchester and Montana], P2) Valio Ltd., P3) Suomen Unilever Oy DiverseyLever, P4) Helsinki University, Faculty of Veterinary Medicine, P5) University of Iceland, P6) Matforsk, P7) TINE, P8) SIK and P9) Arla FoU.

Table 1. Updated contact addresses of members in P96049 "Evaluation of cleaning agents and disinfectants for use in dairies: Methods and mechanisms".

Dr Gun Wirtanen
VTT Biotechnology
P.O. Box 1500 (Tietotie 2, Espoo)
FIN-02044 VTT, Finland
Phone: +358 9 456 5222
Fax: +358 9 455 2103
E-mail: gun.wirtanen@vtt.fi

Satu Salo
VTT Biotechnology
P.O. Box 1500 (Tietotie 2, Espoo)
FIN-02044 VTT, Finland
Phone: +358 9 456 7121
Fax: +358 9 455 2103
E-mail: satu.salo@vtt.fi

Irma Klemetti
Valio Ltd.
P.O. Box 38 (Juustolantie 15)
FIN-73101 Lapinlahti, Finland
Phone: +358 10381 6634
Fax: +358 10381 5326
E-mail: irma.klemetti@valio.fi

Jarmo Juurinen
Valio Oy, Herajoen Meijeri
P.O. Box 116 (Meijeritie 10)
FIN-11101 Riihimäki, Finland
Phone: +358 10381 4262
Fax: +358 10381 4026
E-mail: jarmo.juurinen@valio.fi

Kai Ahlgren
JohnsonDiversey
P.O. Box 311 (Artturinkatu 2)
FIN-20101 Turku, Finland
Phone: +358 2 269 7216
Mobile: +358 400 828 630
Fax: +358 2 230 6726
E-mail: kai.ahlgren@johnsondiversey.com

Urban Wiik
JohnsonDiversey
Hitsaajankatu 4A, FIN-00810 Helsinki, Finland
Phone: +358 9 759 0650
Mobile: +358 400 624 575
Fax: +358 9 781 238
E-mail: urban.wiik@johnsondiversey.com

Dr Solveig Langsrud
Matforsk
Osloveien 1
NO-1430 Ås, Norway
Phone: +47 64 97 01 82
Fax: +47 64 97 03 33
E-mail: solveig.langsrud@matforsk.no

Jens Petter Homleid
TINE FoU-sentret
Boks 7 Kalbakken
NO-0902 Oslo, Norway
Phone: +47 22 93 85 17
Fax: +47 22 93 85 99
E-mail: jens.petter.homleid@tine.no

Dr Monika Neuman
SIK
P.O. Box 5401 (Frans Perssons väg 6)
SE-402 29 Göteborg, Sweden
Phone: +46 31 335 5600
Fax: +46 31 83 37 82
E-mail: mn@sik.se

Ulla Olofson
SIK
P.O. Box 5401 (Frans Perssons väg 6)
SE-402 29 Göteborg, Sweden
Phone: +46 31 335 5675
Fax: +46 31 83 37 82
E-mail: uo@sik.se

Harriet Alnås
Arla Foods Innovation
Box 5407
SE-40229 Göteborg, Sweden
Phone: +46 8 789 50 00
Fax: +46 31 703 2310
E-mail: harriet.alnas@arlafoods.com

Birgitta Axelsson
Arla Foods Koncernmiljö
Box 1508
SE-351 15 Växjö, Sweden
Phone: +46 470 963 00
Fax: +46 470 198 21
E-mail: birgitta.axelsson@arlafoods.com

new agents should be effective in both releasing soil from processing surfaces and killing microbes. The specific topics of the project were:

- development of methods for evaluation of microbial resistance against disinfectants
- evaluation of efficiency of fogging and footbath disinfection
- development of methods for study of cost, usage and effects on environment
- evaluation of cleaning chemicals on spore-contaminated surfaces in flow systems
- evaluation of the efficiency of disinfectants on spores
- evaluation of methods for practical measurement of process hygiene
- development and optimization of environmentally less harmful cleaning agents
- development of test methods for analysing efficiency of cleaning agents
- testing of efficiency of cleaning agents and disinfectants with and without soil
- microbial evaluation of cleaning procedures at pilot scale.

A list of publications, oral and poster presentations as well as theses carried out in the project is given in Appendix 3. The summary of the project was translated into Swedish (Appendix 4), Norwegian (Appendix 5), Finnish (Appendix 6) and Icelandic (Appendix 7) languages.

2. EVALUATING THE EFFICACY OF SANITATION PROCEDURES

2.1 LITERATURE REVIEW IN DAIRY HYGIENE

2.1.1 Soiling mechanisms

The tendency of microbes to adhere to and colonize inert food contact surfaces is a matter of concern in the food-processing industry due to the significant health consequences that can arise from the relatively low numbers of microbes remaining on such surfaces after cleaning (Mattila-Sandholm & Wirtanen, 1992; Carpentier & Cerf, 1993). Different types of layers can form on surfaces in dairy manufacturing plants. Biofilms and biofouling are 2 terms used to describe surface accumulation of organisms. Biofilm is a generic term for positive and negative implications of microbial adhesion. A biofilm is an aggregation of microbial cells and their associated extracellular polymeric substances, actively attached to, growing and multiplying on a surface (Flint *et al*., 1997). Biofouling contains both biofilm and organic soil. The term biofouling describes instances in which biologically active films are considered deleterious (Zottola & Sasahara, 1994), while fouling is used for thin milk component layers formed inside processing equipment (Visser, 1997). Fouling is the major problem encountered in dairies, making cleaning efficacy more difficult and thus resulting in additional costs (de Jong, 1997; Visser & Jeurnink, 1997). Fouling in heat exhangers reduces heating efficacy. Contamination problems caused by biofouling can be solved with regular cleaning (Holah & Gibson, 1999). Biofilms on dairy processing lines are characterized by rapid development (<12 h) and the predominance of single species of bacteria, e.g. *Streptococcus thermophilus* or *Bacillus* spp. (Flint *et al*., 1997). The base of the biofilm formed on gaskets removed from dairy pipelines consisted of nonviable Gram-negative cells, while the outer surface of the biofilm consisted of healthy Gram-positive cocci (Austin & Bergeron, 1995). Sasahara and Zottola (1994) observed that the Gram-negative bacterium *Pseudomonas fragi* can act as a primary colonizing microbe that may entrap *Listeria monocytogenes*. It has been observed that one heat-resistant microbe, *Streptococcus thermophilus*, can adhere to the pasteurized milk section of a pasteurizer, inoculating the milk at a rate of 10^6 cells/ml (Carpentier & Cerf, 1993).

The soil to be removed consists mainly of milk residues, which include fat, proteins, lactose and milk stone (Kessler, 1981). Milk stone begins to form when milk is heated above 60 °C. The deposits adhere tightly to the surfaces, and after runs of more than 8 h a change in colour from whitish to brownish can also be observed (Bylund, 1995).

Surface irregularities such as roughness, crevices and pits have been shown to increase bacterial adherence by both increasing bacterial cell attachment and decreasing removal of attached cells by cleaning (Characklis, 1981). Regions of the gaskets that make the seal with the pipe were shown to be more heavily colonized than the inner diameter of the gasket (Austin & Bergeron, 1995).

2.1.2 Pathogenic microbes in dairy processes

Milk used for milk products is normally pasteurized (15–20 s, 72–75 ºC). Some cheese products are made of milk that is just heated (≥ 15 s, 63–65 ºC). Pasteurizing eliminates most of the pathogens and spoilage organisms from milk (Chapman & Sharpe, 1990). Post-contamination may occur, if proper levels of hygiene are not maintained in the dairy. Pathogenic bacteria found in milk and milk products include *Escherichia coli*, *L. monocytogenes*, *Yersinia enterocolitica*, *Staphylococcus aureus*; *Salmonella* sp. and *Bacillus cereus* (Ahmed *et al*., 1983b; Johnson *et al*., 1990; Zottola & Smith, 1991; Zuniga-Estrada *et al*., 1995; Benkerroum *et al*., 2002). Some examples of pathogens found in dairy products are listed in Table 2 (Hasting, 1995).

Table 2. Examples of pathogens found in dairy products (Hasting, 1995).

Year	Country	Product	Number of reported ill persons	Causative organism	Estimated cost (£)
1965	USA	cheese	42	*Staphylococcus aureus*	250 000
1985	USA	cheese	142 (47 deaths)	*Listeria* sp.	411 000 000
1989	UK	hazelnut yoghurt	27 (1 death)	*Clostridium botulinum*	220 000

Bacillus cereus in dairy products

B. cereus occurs widely in dairy products. Ahmed *et al.* (1983b) found that 9% of raw milk, 35% of pasteurized milk, 14% of cheese and 48% of ice-cream samples were contaminated with *B. cereus*, while Wong *et al.* (1988) isolated *B. cereus* organisms from 29% of milk powder, 17% of fermented milk, 52% of ice-cream, 35% of soft-ice and 2% of pasteurized milk samples. Studies performed in Scotland showed that 75% of pasteurized milk samples contained spores of *B. cereus* (Davies & Wilkinson, 1973). However, food-poisoning outbreaks caused by dairy products contaminated with *B. cereus* have been rare (Wong *et al*., 1988).

Escherichia coli in dairy products

Raw milk often contains *E. coli,* which is killed in the pasteurization process, and products can be contaminated due to poor production hygiene. *E. coli* has been detected in fresh soft cheeses, Camembert and Brie cheeses. However, food-poisoning outbreaks caused by dairy products contaminated with *E. coli* have been rare (Heeschen & Hahn, 1996). *E. coli* O157:H7 has been isolated from raw milk and bulk tank milk samples in the range of 0 to 10% (Hancock *et al.,* 1994; Keene *et al.,* 1997; Mechie *et al.,* 1997; Murinda *et al.,* 2002). The results obtained by Murinda *et al.* (2002) however show that incidences of *E. coli* O157:H7 are more often associated with undercooked ground beef than with the consumption of raw milk.

Listeria monocytogenes in dairy products

L. monocytogenes has been found in cheese, milk, ice cream and butter (Zuniga-Estrada *et al*., 1995; Benkerroum *et al*., 2002). The main contamination routes are surfaces of process equipment on which biofilm containing *Listeria* can form (Farber & Peterkin, 1991; Farber *et al*., 1992; Miller *et al*., 1997). *Listeria* is known to survive high salt concentrations. Its survival during pasteurization treatment varies (Donnelly & Briggs, 1986; Mackey & Bratchell, 1989; Lovett *et al*., 1990; Bradshaw *et al*.,1991; Farber & Peterkin, 1991; Farber *et al*., 1992; Donnelly, 1994).

Staphylococcus aureus in dairy products

S. aureus is another pathogen of concern in dairy products. Its behaviour in sour milk products such as yoghurt is worthwhile to investigate due to its precence in raw milk in relativeley high numbers and its incidence on human health (Benkerroum *et al*., 2002). Accoding to studies by Benkerroum *et al*. (2002) staphylococcci grew rapidly during the initial fermentation. Similar behviour of *S. aureus* has previously been reported both in yoghurt and cheese (Ahmed *et al*., 1983a; Attaie *et al*., 1987). A recent survey revealed that *S. aureus* was involved in 15% of recorded foodborne illnesses caused by dairy products in eight developed countries whereas *L. monocytogenes* was involved in 22% (Benkerroum *et al*., 2002).

2.1.3 Sanitation procedures

Biofilm control currently relies on the effectiveness of cleaning-in-place (CIP) systems (Flint *et al*., 1997). A CIP programme for a pasteurizer circuit normally consists of prerinsing, circulation of an alkaline detergent solution, intermediate water rinse, circulation of acid solution and post-rinsing. Acid circulation is included to remove encrusted protein and salts from the surfaces of heat-treatment equipment. Prerinsing is normally performed with cold water. Alkaline circulation is performed for 15–20 min with hot (75–80ºC) water containing 1.0–1.5% alkaline detergents (Chisti & Moo-Young, 1994). After an intermediate water rinse acidic circulation is performed for 5 min with hot (65–70 ºC) 1% acid solution. The flow rate must be more than 1.5 m/s to achieve a mechanical force that effectively prevents biofilm build-up (LeChevallier *et al*., 1990). Austin and Bergeron (1995) suggested that extensive bacterial biofilms may develop on gaskets present in various areas of dairies even after regular CIP procedures.

Parts of equipment and other utensils such as cheese moulds and transportation frames for milk cartons can be washed in closed washing tunnels. The utensils are transported to the washer with conveyors, and nozzels in the tunnel shower the equipment under light pressure (< 2 bar) using large volumes of detergent solution (Daufin *et al*., 1987). Ultrasound cleaning can also be used to clean utensils (Kivelä, 1996), while open surfaces can be cleaned using foam cleaning (Hansen, 1986).

2.2 HYGIENE OF FOOTBATHS

2.2.1 Use and control of footbaths

In food production, two of the measures used to prevent cross-contamination from ‘dirty’ areas to areas of higher hygienic levels are change of footwear and use of disinfecting footbaths. The efficacy of disinfecting footwear is not well documented, and little is known about what types of disinfectants are most suitable for disinfecting footbaths. The ideal disinfectant to use should be effective also when soil is left in footbath. It should also be harmless to people, footwear and the floor material and it should combat biofilm formation with no resistance build-up in the microbes in the footbath. In the worst case, the disinfecting footbath could represent a contamination problem with resistant, biofilm-forming bacteria that may be transferred to the production area by the footwear.

Some bacteria have natural properties that enable them to circumvent the action of a disinfectant, while others have acquired resistance mechanisms from other bacteria (McDonnell & Russell, 1999). Such bacteria may be selected in disinfecting footbaths. In addition, bacteria can become adapted to tolerate higher concentrations of a disinfectant, thus making sensitive strains into resistant ones. Bacterial growth in aqueous solutions of disinfectants has been reported (Lowbury, 1951; Heinzel & Bellinger, 1982) and footbaths could thus serve as a contamination source.

It has been demonstrated that bacteria may develop resistance to tenside-based disinfectants, such as tetraethyleneglycol orthophtalate (TEGO) compounds or quaternary ammonium compounds (Block, 1991). Kellet (1979) isolated bacteria from working-strength solutions of TEGO in an animal clinic; the bacteria were tentatively identified to belong to the genus *Acinetobacter*. Gram-negative bacteria such as *Pseudomonas* spp. and coliforms often have relatively high resistance to quaternary ammonium compounds and may develop higher resistance if exposed to subinhibitory concentrations of these disinfectants (Russell & Chopra, 1996). Survival after disinfection with oxidative compounds such as chlorine or peracetic acid (CH_3COOOH) is more often explained by biofilm formation (Bolton *et al.,* 1988; Clark *et al.,* 1994).

A questionnaire was distributed to 30 Norwegian dairy plants using footbaths. They were asked the type and number of footbaths, disinfectant used (type, concentration) and routines for use (refilling, change of disinfectant). The number of footbaths in each dairy varied, as did the area of the footbaths (0.17–1.8 m^2) and the depth (2.5–12 cm). The mean amount of disinfectant used varied 5–15 l/m^2. The most commonly used disinfectant in the footbaths was hypochlorite; 20 out of 30 dairies used it (Table 3). TEGO and TP-99 were used by 8 and 6 dairies, respectively. One dairy alternated between the amphoteric tenside Betane and the quaternary ammonium compound Veterinær Ultra Des and one used the peracetic acid-based Oxonia Aktiv.

Table 3. Types of disinfectant used in footbaths in 30 Norwegian dairies. Some of the dairies used 2 disinfectants.

Disinfectant type	Number of dairies
Hypochlorite	20
Hydrogen peroxide (H_2O_2) /Peracetic acid (CH_3COOOH)	1
Amphoteric tensides	8
Cationic tensides + Amphoteric tensides[1]	1
Alkyaminoacetate	6

[1] Rotational use of 2 types of disinfectant in the same footbath.

Eleven out of the 30 dairies contacted participated in the extended study to determine the bacteriological status of the footbaths. In these dairies a total of 83 footbaths were used and of these 46 were sampled (Table 4). The production management received a sampling kit and instructions for sampling (see Chapter 5.5).

The samples often contained spore-forming bacteria. They were not analysed further, since bacterial spores were expected to survive in the concentrations of disinfectants used. Vegetative bacteria were isolated from 10 out of 14 footbaths with hypochlorite. Bacteria isolated from used chlorine compounds were identified as *Acinetobacter*-like, *Staphylococcus*-like or unknown. The bacteria were found both in the disinfectant (6 samples) and on the surfaces (9 samples). Ten out of 11 footbaths containing TEGO demonstrated bacteria, both surviving in the disinfectant and on the surfaces. Footbaths with TEGO 103G appeared to select for *Pseudomonas, Cedecea, Serratia* or *Proteus* resistant to both TEGO

103G and other surface-active disinfectants e.g. benzalkonium chloride ($C_6H_5CH_2(CH_3)_2NRCl$). We therefore question the effectiveness of TEGO 103G as a footbath disinfectant. Alternation with TP-99 in one dairy did not improve the efficacy. No bacteria were isolated from 3 footbaths with 3% TP-99; however, in footbaths with lower concentrations (1–3%), 8 out of 10 were positive for bacteria in suspension and on surfaces. In summary, bacteria were isolated from about 75% of the footbaths tested and none of the disinfectants totally prevented bacterial survival. Isolation of viable bacteria from the disinfectant used indicated that the disinfectant was neutralized by soil or that bacteria had developed resistance to the disinfectant applied. It is difficult to compare the efficacy of different disinfectants due to differences in routines and the level of contamination. Depressions and scars from wear and tear of the footbaths may influence survival of bacteria as well. The concentration used, and the frequencies of emptying and refilling also influenced the survival of microbes in the footbaths (Appendix 8).

Table 4. Number of footbaths with bacterial growth.

Disinfectant	Number of footbaths	In the disinfectant	In the neutralized disinfectant	Swab before rinsing of the footbath	Swab after rinsing of the footbath
Chlorine compounds[1]	14	3	7	10	10
TEGO[2]	11	9	10	9	9
TP-99[3]	13	5	8	8	8
Betane[4]	1	0	0	0	0
Oxonia Aktiv[5]	4	0	1	0	1
Veterinær Ultra Des[6]	3	0	1	0	0

[1] Disinfectants containing hypochlorite
[2] Based on amphoteric tensides in the TEGO group
[3] Based on alkylaminoacetate
[4] Based on amphoteric tensides
[5] Based on hydrogen peroxide and peracetic acid
[6] Based on quaternary ammonium compound.

Reports on the isolation of bacteria from disinfectants and equipment used for disinfection have long been published (Lowbury, 1951; Heinzel & Bellinger, 1982). Therefore, it is not surprising that we isolated bacteria from footbaths and

that the disinfectants sampled often contained heavy microbial loads. The use of footbaths may kill most bacteria, but some will survive and increase the risk of bacteria spreading from footbath to floors in the critical zones and to the environment with aerosols in locations where the footbath is emptied, rinsed and refilled. The use of footbaths also results in additional wetness on floors. Bacteria on the footwear will be protected due to higher survival rates of attached bacteria (Langsrud & Møretrø, 2001). It is important to remember that the majority of the strains isolated were easily killed by the disinfectant after laboratory cultivation. This has also been noted earlier when cultivating bacteria isolated from disinfectants and disinfection equipment (Lowbury, 1951; Carson *et al.,* 1972; Heinzel & Bellinger, 1982). Therefore, bacteria considered sensitive to disinfectant, e.g. *L. monocytogenes*, could survive and spread from footbaths.

The main objective of this study was to obtain information on the use of footbaths in the Norwegian dairy industry and to propose methods for hygienic control. We have documented the need for more effective hygienic control by determining the occurrence and location of bacteria in disinfecting footbaths. Although bacteria on the footwear could be killed by the disinfectant, a change in footwear when entering a critical zone is recommended as a more effective hygienic measure and should always be carried out when entering a high-risk area. For those dairies that prefer to continue the use of footbaths as a single measure or in combination with change in footwear, we recommend that the concentration of disinfectant be higher than that in general use and that both the concentration and the frequencies of refilling be documented. A bacteriological control should also be included in the routines; this could easily be done by swabbing about 10 cm^2 of the footbath after emptying. If a consistent biofilm is developed, the purchase of a new footbath using higher concentrations is needed.

2.2.2 Hygienic conditions of footbaths

Footbaths were tested in a Swedish dairy in a way similar to that done in Norway. A chlorine compound was used as a disinfectant at a recommended concentration of 350 ppm. Samples were taken from all 8 footbaths in the dairy on 3 different occasions, and from one selected footbath the sampling was carried out on 14 different days during a month. All the samples were taken from the corner of the footbath farthest from the site where the disinfectant was added and at the same spot each time. The samples were taken with swabs and spread

on a Petri dish with plate count agar (PCA) and another with blood agar. The Petri dishes were incubated at 20 ºC, 5–7 d nd 3–5 d, respectively.

The concentration of chlorine was tested on the same occasion. The chlorine concentration varied from zero to 1000 ppm and there were no correlations between the concentration and numbers or types of microbes. The test showed that the footbaths contained many microbes of different genera, e.g. moulds, yeasts, *Enterobacteriaceae*, *Pseudomonas*, *Bacillus* and *Micrococcus*.

The conclusion reached is that the footbaths did not function in the way they were intended, probably because biofilms may have formed on the walls of the footbath and that neither the disinfectant nor the cleaning routines used may have been effective enough. The question of whether the footbath was more harmful than useful was raised. Suggested improvements included better control of the chlorine dosage and, if necessary, use of another type of disinfectant as well as cleaning of the footbaths at regular intervals.

2.3 Fogging Procedures in Dairies

2.3.1 Control of fogging disinfection

Fogging disinfection is finely dispersed droplets (fog) of a disinfectant within a room. The purpose of fogging disinfection is to ensure that all regions and equipment in the room have received an adequate application of the disinfectant (Burfoot *et al.*, 1999). Fogging systems are costly, but could be cost-efficient and also result in improved hygiene if used appropriately. However, whether or not the the systems always function as expected is an open question. Earlier, it has been shown that fogging disinfection can reduce the microbial counts in both air and on surfaces, but the efficacy will be dependent on the droplet size, temperature and humidity in the room, concentration and contact time of the disinfectant, thoroughness of the washing process etc. (Burfoot *et al.*, 1999).

The efficacy of disinfectants is most often tested in laboratories with laboratory grown microbes, using standardized methods. A method to test fogging disinfection has been proposed with laboratory-grown microbes dried on stainless steel (Burfoot *et al.*, 1999). However, it has been shown that slow-growing bacteria in microcolonies or as biofilm growth are more difficult to

eliminate (Morton *et al.,* 1998). Therefore, the efficacy of the fogging disinfection system must also be evaluated in the plant after installment.

The 5 cheese plants included in this survey used fogging disinfection systems from Henkel Ecolab AS, Norway, or mobile equipment from Arcon AS, Norway (Clean Tech aps, Denmark) installed. The effect of fogging disinfection was measured using contact agar plates. A total of 10–19 control points were sampled, before and after disinfection using contact plates with PCA. The plates were incubated at 20 °C for 7 d before counting. Examples of control points included walls, ceilings, conveyor belts, electric switches, packing machines, ventilation ducts (outside) and tanks (outside). The controls included samples from different heights and undersides of objects. Samples were taken at different distances from the nozzle. The microbial controls were taken in the evening before disinfection and after disinfection in the morning just before work began.

An overview of the microbial counts is given in Tables 5 and 6. Plant 1 was not audited and the efficacy of washing is not known. TP-99 was used for disinfection. Microbes appeared to be reduced in 6 out of 9 control points in Plant 1. The control points without reduction were places that were difficult to reach, e.g. behind shelves, under a washing machine and on the wall behind the washing machine. Yeast and spore-forming Gram-positive rods (possibly *Bacillus*) were found in the microflora isolated after disinfection.

The washing procedure in Plant 2 was very exhaustive and the room was relatively humid and cool after full washing. The room was visibly filled with disinfectant fog during disinfection with peracetic acid-based Oxonia Aktiv. The plant alternated with alkylaminoacetate-based TP-99. The plant used an automatically operated unit and the interval between disinfection and rinsing was for unknown reasons omitted. The contact time was reduced from the planned 20 min to the fog-producing period. The fogging disinfection appeared very efficient, with reduction at all control points. However, some bacteria survived on a water hose, wallboard joints and on the underside of a table. The microflora after disinfection consisted of slowly growing pink colonies identified as *Methylobacterium* (identification based on fatty-acid content and 16S rDNA analysis) as well as slimy colonies of a Gram-positive bacterium identified as *Rhodococcus* sp. by 16S rDNA analysis.

The washing process in Plant 3 was exhaustive except for a conveyor belt which was not loosened, washed or dried on the underside. The room was humid and warm after the washing process and visibly filled with disinfectant fog during disinfection. However, the fog could not be expected to penetrate on the under side of the transport band. Less fog was also seen near the ceiling in Plant 3 compared with Plant 2. The agents used were a alkylaminoacetate-based disinfectant, TP-99, alternated with a disinfectant based on hydrogen peroxide and peracetic acid, Oxonia Aktiv. The disinfecting efficacy was also high in Plant 3, having only one control point with less than 50% reduction in viable counts. The microflora consisted of small red and yellow colonies and also different moulds after disinfection. The microflora under the conveyor belt mainly consisted of Gram-negative bacteria.

Only equipment and tables used during the day of the audit were washed in Plant 4. All surfaces were visibly clean and the room was relatively dry with normal temperature after washing. The fogging system did not function as planned in Dairy 4, because the droplet size was too large to make efficient fog. The fog produced was mainly concentrated around, and precipitated close by, the nozzles. The disinfectant used was Oxonia Aktiv alternated with TP-99. The contact agar plates taken at the 15 control points had visible growth before disinfection, with reduction on 10. The microflora consisted of yeast, different moulds and bacteria (yellow, pink and colourless colonies). The pink and yellow colonies appeared after approximately 1 week.

The washing process in Plant 5 was mainly performed using an automatic CIP system. The exterior of the equipment, walls and packing machines were not thoroughly washed but all surfaces were visibly clean. The room was relatively dry and with normal temperature. The quality of the fog produced appeared normal, but it was concentrated around the 2 nozzle holders. The majority of production equipment and packing lines were located in the fog, but equipment and tanks were also located elsewhere in the production hall. The disinfectant used was the amphoteric tenside-based Betane (Arcon AS) and the fogging disinfection was mainly used to control the quality of the air at the premises. Only 10 control points were sampled before and after disinfection at Plant 5, all of them only in direct contact with the product. None of the 7 control points with growth before disinfection indicated full reduction in viable counts, although one point indicated about 50% reduction. The microflora mainly consisted of slow growing small red *Methylobacterium* colonies, Gram-positive spore-forming

bacteria (possibly *Bacillus* sp.), Gram-positive catalase-negative rods (possibly lactobacilli) and slow-growing yellow colonies. In summary, the efficacy of the washing and the extent to which the fog filled the room varied greatly among the dairies, and this was reflected in the microbial counts. About 70% of a total of 75 agar plates had microbial growth (bacteria, yeast or moulds) before disinfection (Tables 5 and 6). Only 2 agar plates had colonies after, but not prior to, disinfection.

Table 5. Total number of samples in each plant and numbers of contact agar plates with and without growth before disinfection. The plates were incubated at 20 °C for 2.5 d and 7 d.

Plant	Samples	No growth[1]	Growth before[2]	Growth after, not before[3]
1	15	5	9	1
2	16	4	12	0
3	19	5	14	0
4	15	0	15	0
5	10	2	7	1

[1] Number of samples without growth before or after disinfection
[2] Number of samples with growth before disinfection
[3] Number of samples with growth after disinfection, but not before.

Table 6. Efficacy of fogging disinfection measured at control points before and after disinfection.

	1–50 colonies before disinfection			> 50 colonies before disinfection		
Plant	Before[1]	Full reduction[2]	Reduction[3]	Before[1]	Full reduction[2]	Reduction[3]
1	4	1	1	5	1	3
2	8	6	2	4	3	1
3	9	7	2	5	4	0
4	1	0	1	14	3	6
5	4	0	0	3	0	1

[1] Number of plates with colonies before disinfection
[2] Number of plates with no visible growth on plates after disinfection
[3] Reduction of colony counts >50%.

The disinfectants are documented using standardized suspension methods, in which killing of more than 99.999% of laboratory-grown microbes are needed. However, this study has shown that bacteria can survive after fogging with disinfectants. Few isolates were highly resistant to the in-use concentration of oxidizing disinfectants. The slow-growing bacterium isolated in several plants was resistant to tenside-based disinfectants if grown under nutrient limited-conditions. Thus, survival was probably caused by lack of contact between microbes and disinfectant, e.g. insufficient washing process, inefficient fog droplets, too low concentration of disinfectant in the fog or inadequate amount of fog in the room and bacterial resistance to disinfectants.

Even though equipment and environment appeared to be clean by visual inspectin, attached microbes may have formed microcolonies or excreted protective components around the cells. It is well known that attached microbes are more resistant than planktonic, and that biofilm-growth impedes the killing of microbes. Therefore, it is not surprising that the most effective disinfection was seen in plants with an exhaustive washing process before disinfection. The spore-forming bacteria isolated after fogged, but not washed surfaces, could have been reduced if an alkaline wash had been carried out prior to disinfection. Langsrud and Sundheim (2000) demonstrated that alkaline washes can potentiate the effect of peracetic acid. Washing with alkaline solutions not only removes dirt and microbes, but may also sensitize microbes to disinfection.

Detection of surviving microbes in the environment after fogging with contact agar plates is an easy, but relatively insensitive method for the control of fogging efficacy. About 15–20 different sites could be selected to document the efficacy of the disinfection and to designate fewer, but important, control points. Disinfectant fog should affect airborne microbes; thus, in addition to surface control, air sampling could give valuable information. If the target is to improve air quality, all possible sources contributing to the air microflora should be evaluated. It should be remembered that microbes survive, but do not grow in dry environments. Frequent use of fogging in rooms that are mostly dry, e.g. for closed production or packaging, could result in longer periods under humid conditions with a possibility for microbial survival and biofilm growth.

The results suggest that the technical performance of the fogging disinfection should be monitored frequently to ensure optimal disinfection. The highest efficacy of the fogging disinfection on environmental surfaces was seen in plants with an exhaustive washing process preceding the disinfection. Our results demonstrate that visual and microbial control can be an effective tool enabling improved hygiene. Critical control points should be identified both for technical performance of the fogging system and disinfection efficacy. Based on these results we recommend:

- regular control of the nozzle and complete filling of the room with fog,
- regular control to ensure that the disinfection programme is functioning as planned,
- regular control of the amount of disinfectant consumed,
- random sampling of the disinfectant concentration in the fog and after rinsing,
- monitoring disinfection efficacy,
- auditing of the washing programme preceding disinfection and evaluation of the washing process,
- random sampling using 15–20 different control points as well as air sampling. The fogging system could be optimized until the necessary level of disinfection is attained,
- regular control using a few control points to show that the hygienic performance of the system is maintained.

2.3.2 Air disinfection

To be able to evaluate air disinfection efficiency, a method given in Chapter 5.7 has been developed. The method gives repeatable values for the 3 replicates used in each trial. One drawback with the method may be that the microbes in this method are in a very dry state, more dry than under normal conditions, and may therefore be more resistant. *Candida albicans* appeared to be sensitive to drying, because a high number of organisms was killed during overnight drying. It was also noticed that the mould spores should be stored below 8°C to maintain them as spores. When the stainless-steel coupons inoculated with *Aspergillus niger* spores were placed into the cheese storage room the fungal spores started to germinate during the ozone treatment, if the temperature in the room was elevated to approximately 20 °C. Using all 4 microbes (*C. albicans, A. niger, B.*

cereus and *S. aureus)* in the same trial emphasizes the reliability of the results achieved. The placement of the stainless-steel coupons appears to affect the result. A slightly higher reduction was found on the coupons on top of the shelf. The results show wide differences between the 2 agents used.

Air disinfection using ozone

The disinfection efficiency of ozone was not good. The numbers of *Staphylococcus aureus* and *B. cereus* were not reduced at all with either 1 ppm or 2 ppm for 6 h (results for 1 ppm not shown). The number of *A. niger* spores was only marginally i.e. <0.5 log-units reduced. C. *albicans* was affected by ozone with a reduction up to 1 log-unit. The position of the stainless-steel coupons did not appear to affect the result (Fig. 3). However, due to the low reduction in number of microbes it is difficult to say anything about the influence of the position of the coupons on the reduction.

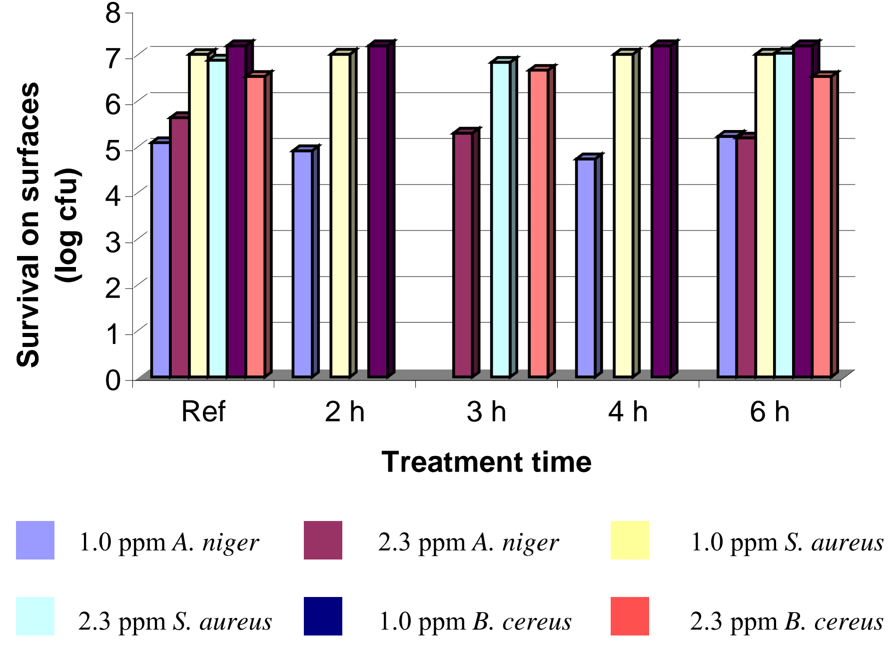


Figure 3. Microbial growth on the stainless-steel plates after air disinfection.

Preliminary experiments, in which *B. subtilis, Micrococcus luteus, E. coli* and yeast were grown on agar plates and the agar plates were treated directly with ozone, showed that the microbes were killed. To verify these results, microbes growing on stainless-steel coupons were removed from the hard surface through shaking in a neutralizer for 30 min and various dilutions were pipetted onto agar plates. These agar plates were finally kept in a cheese storage room during air disinfection. A reduction of all 4 microbes was noticed. The reduction in *C. albicans* was approximately 3 log-units and for *S. aureus* approximately 4 log-units. However, all the surviving microbes were found near the edge of the coupon and the edge could have prevented the ozone from reaching the agar plate. The number of *B. cereus* spores and *A. niger* spores were in this case reduced by 1 log-unit.

Air disinfection based on fogging

When using an effective hydrogen peroxide-based agent, the reduction with fogging varied between 4 and 7 log-units for the vegetative cells and 0.5–1.0 log-units for spores, (Fig. 4a). No significant reduction was found using a less effective tenside-based agent (Fig. 4b).

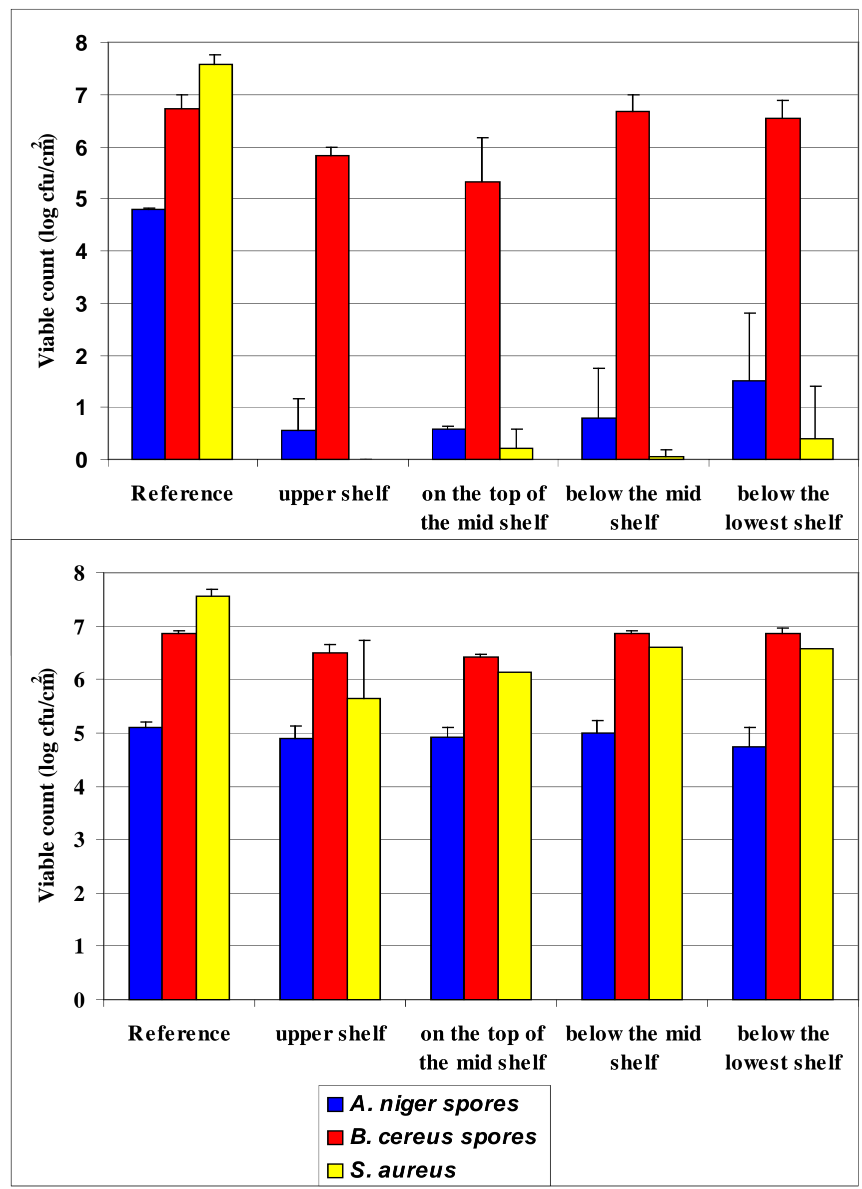


Figure 4. Air disinfection of surfaces contaminated with spores of Aspergillus niger and Bacillus cereus as well as vegetative cells of Staphylococcus aureus using fogging with a) an effective hydrogen peroxide-based agent and b) a less effective tenside-based agent.

2.4 Hygiene in an Open Dairy Process – Cheese Production

2.4.1 Cheese production

Cheeses have been made for many centuries to preserve the milk. Cheese is a concentrate of milk comprising casein, particles soluble in water and milk fat. As a by-product whey is formed, since the casein and fat are concentrated into cheese. Some examples of cheese types are ripened hard or semihard cheese, blue cheese, unripened cheese as well as whey cheese. The use of rennets, cooking of the cheese matrix and forming under pressure in moulds are common phases in the production of most cheese types (Fox, 1987).

There are many crucial phases in the production of semihard and hard cheeses. According to Frandsen (1992) the control procedure during production includes analysis of the milk content, determination of the mammalian cell number, bacterial count and freezing point as well as possible residues of antibiotics. The cheese-milk is heat-treated either by pasteurization (70–72 ºC/15–20 s) or by heating (65 ºC/15 s), homogenized and preacidified using a starter culture typical for the cheese type produced. The heat treatment is performed to ensure that neither spoilage microbes nor pathogens such as *L. monocytogenes* are transferred from the raw milk into the cheese (Bertrand, 1987; Chapman & Sharpe, 1990; Frandsen, 1992). During acidification rennet is added to the cheese-milk, the casein in the milk is precipitated and the cheese structure is formed. The jelly stucture or curd is cut into small pieces with special cutting blades charasteristic for each cheese. During the syneresis phase whey is worked out of the granular curd and the whey and granules are separated from each other.

The cheese moulds are used to remove the last part of the whey from the cheese curd under pressure. In this phase the characteristic shape and surface of the cheese is formed. Its main functions are to obtain a certain consistency, promote ripening and adjust the amount of whey secreted (Bertrand, 1987; Chapman & Shape, 1990). In earlier days cheese cloth and wooden moulds were used to form the shape and skin of the cheese. At present these moulds are mostly made of plastic. They are convenient because they are strong, light, cleanable, decrease the process noise and are suitable for automation (Tamine, 1993). Moulds typical for each type of cheese are used to obtain the characteristics of the particular cheese type (Anon., 1980).

2.4.2 Methods in evaluation of cheese mould cleanliness

The design of cheese moulds is unfortunately not very hygienic. The moulds are perforated by small holes for drainage, and made of a plastic material that cannot withstand temperatures higher than 70 °C and their surface finish will in time be quite rough. Therefore, it is important to have cleaning and disinfecting procedures that are effective enough to ensure safe cheese production. The aim here was to select and improve methods for the evaluation of washing and disinfection effect of cheese moulds. These experiments have been performed on cheese-moulds on 5 different occasions. Two methods, of the original 8 given in Chapter 5.6 were selected. These 2 methods, washing out and TTC-method, measure the remaining numbers of living microbes. We are continuing to search for an effective method for measuring the remaining protein fraction. Four different cleaning agents were tested.

The results using Check Pro protein kits are greatly dependent on the pH, leading to possible false-negative reactions. Therefore, to determine whether Check Pro is a useful method, the protein measurements should be repeated with a neutral pH in the rinsing water. Macroscopic ultraviolet (UV) illumination was also difficult in combination with the plastic material in the cheese moulds. UV illumination was evaluated in 2 trials and then rejected. It may be a potentially useful method after additional training and improvements in application. It was difficult to use the direct epifluorescence microscopy (DEM) technique on cheese moulds. Some bacterial cells were seen but no quantification was possible.

Swabbing showed the same effects as washing out, but with much lower recovery of bacteria. We chose washing out as the better of these 2 methods. The Bioscreen measurements showed the same trends as the other culture techniques, but with quite large standard deviations between trials as well as between replicates within the same trial. After 4 trials the Bioscreen was rejected in favour of the 2 other culturing-based techniques: washing out and TTC (Table 7). The adenosinetriphosphate (ATP) measurements are only relevant for lactic acid bacteria because the spores do not contain ATP. The values obtained after cleaning, however, were very low and it is doubtful if ATP measurements are sensitive enough in this application.

Table 7. Results obtained in full-scale cheese mould studies.

Strain	Sample	TTC moulding		Washing out (cfu/mould)	
		Mean	St. Dev.	Mean	St. Dev.
Bacillus	Reference	5.0	0.0	7.5	0.0
	SU436	2.5	0.0	4.6	0.1
	Horolit CIP	**2.1**	**0.2**	4.6	0.1
	P3 VR	2.4	0.4	4.5	0.0
Lactic acid bacteria	Reference	4.0	0.0	6.6	0.0
	SU436	1.6	0.6	2.5	0.1
	Horolit CIP	**1.2**	**0.6**	2.4	0.3
	P3 VR	1.4	0.6	2.5	0.2

The washing out technique gave much higher numbers of bacterial cells than traditional swabbing. It appears to be a simple and promising method. Moulding with TTC is also promising and quite simple to perform. Visual inspection is even more sensitive than when using a camera. The camera, however, is simple and fast to use and gives a quantitative value of the result.

We conclude that the methods ATP, swabbing, washing out, and moulding with TTC, all indicated that the traditional agent (Horolit CIP) was more efficient in cleaning than the others tested. When using an effective cleaning agent and lactic acid bacterial contaminants, no growth could be detected after washing. The *Bacillus* strain used did show some growth, which is logical since the spores survive and adhere better than lactic acid bacterial cells. Comparing cleaning and killing for different cleaning agents using the washing out method gave results that were consistent for both organisms and during all repetitions. The results from the TTC method correlate with the washing out method. Similar results are also achieved when both indicator organisms were used.

2.4.3 Ultrasound cleaning procedure in cheese production

Narrow holes in the cheese mould walls serve for removal of the whey (Kivelä, 1996). These holes penetrate the walls and through them the whey is removed during the pressing phase. When the whey flows through the narrow cavities and perforated holes, the mould surface becomes soiled and later the whey can no longer penetrate through these holes, thus lowering cheese quality. Furthermore, the cheese material left on the surface also functions as a good substrate for microbial growth (Chapman & Sharpe, 1990; Kivelä, 1996). Clogging of the cavities in the moulds interferes with removal of the whey and the cheese structure remains soft (Koivisto, 1999).

Cleaning of cheese moulds using low-pressure cleaning or common washing machines is not applicable in the high-speed cheese-making process. In addition, cleaning methods based on high temperatures and long-lasting soaking in detergent solutions cannot be afforded in cheese manufacturing. To improve cleanability of the plastic moulds washing machines based on ultrasonics have been developed. In ultrasonic-based cleaning the target material is soaked in a washing solution and a strong ultrasonic field is created by special ultrasonic elements. The washing properties are dependent on the amplitude and frequency of the ultrasound as well as on the depth of the washing solution, the external pressure and the temperature (Anon., 1999). Disintegration of the dirt is based on the breaking up of gas bubbles in the fluid (Kivelä, 1996). Washing based on ultrasonics is smoother compared with other common methods, because the ultrasonic energy is transmitted through the target material (Heino, 2000). Ultrasound cavitation cleans the narrow cavities, thereafter making again possible for the whey to flow through them (Kivelä, 1996). Results obtained in the ultrasound-based cleaning studies performed in this project are presented in Appendices 9–10.

2.5 Hygiene in Closed Dairy Processes – Milk and Yoghurt Production

2.5.1 Yoghurt production

Yoghurt manufacturing is a long process beginning with fixing the fat content of milk to 2.0–3.5%. Milk is fortified by adding nonfat milk solids or concentrated

by evaporation to obtain the final texture desired (Matalon & Sandine, 1986). The basic mix is then homogenized and heat-treated before fermentation (Matalon & Sandine, 1986; Savello and Dargan, 1995). After heat treatment the milk is cooled to 40–45 °C, inoculated with 2% yoghurt bulk culture and incubated until sufficient acidity is attained (Matalon & Sandine, 1986). Consistency of yoghurt is dependent on the starter culture used, storage temperature and addition of stabilizing agents (Sinha *et al*., 1989; Savello & Dargan, 1995). Yoghurt processing is a very demanding task, especially when probiotic bacteria such as *Lactobacillus acidophilus* and *Bifidobacterium bifidum* are used, because the growth rate of these bacteria may differ considerably and affect the fermentation process. Long incubation periods at temperatures around 37 °C are not attainable industrially if milk has not been sterilized in advance. After the heat treatment the facilities in the processing plant must be aseptic to avoid the risk of microbial growth in the final product (Driessen & Loones, 1992).

2.5.2 Cleaning procedures used for pasteurizers

A CIP programme for a pasteurizer circuit normally consists of prerinsing, circulation of an alkaline detergent solution, intermediate water rinse, circulation of acid solution and postrinsing. Acid circulation is included to remove encrusted protein and salts from the surfaces of heat-treatment equipment (Chisti & Moo-Young, 1994). The flow rate must be more than 1.5 m/s to achieve the mechanical force necessary to prevent biofilm build-up (LeChevallier *et al*., 1990). Extensive bacterial biofilms may also develop on gaskets in dairy equipment when regular CIP procedures are performed (Austin & Bergeron, 1995; Storgårds *et al*., 1999).

Long-standing processing will cause fouling on the surface of processing equipment, especially in heat exchanger plates which results in the growth of harmful thermophilic bacteria. It is important to specify the correct processing times and cleaning methods. The cleaning efficiency of different cleaning agents was tested using milk burned for 8 h on stainless-steel coupons (AISI 304, 2B) using the pilot equipment (Figs 5 and 6). Biofilms for cleaning efficiency tests were also formed on the coupons for 4 h (40 l pasteurized milk inoculated with harmful thermophilic bacteria) in the pilot-scale tank-pipeline system Biorig (Tankki Oy, Finland), in which the milk (43 °C) was circulated. Test coupons

were cleaned with 3 different detergents, 0.7% NaOH, 0.7% NaOH containing 0.2% SU 560 (chelator) and 1.5% potassium hydroxide (KOH), all with or without nitric acid (HNO_3) treatment (as single-phase cleaning and a 2-phase cleaning).

The difference between various cleaning programmes can be seen in Fig. 7: stainless-steel surfaces soiled with burned yoghurt milk containing harmful thermophilic bacteria before cleaning (Fig. 7a), after single-phase cleaning with 0.7% NaOH (Fig. 7b), after 2-phase cleaning with 0.7% NaOH and 1.0% HNO_3 (Fig. 7c), after 2-phase cleaning with 0.7% NaOH containing the chelator solution and 1.0% HNO_3 (Fig. 7d) and after single-phase cleaning with 0.7% NaOH containing the chelator solution (Fig. 7e).

The study showed that bacterial numbers of harmful thermophilic bacteria will increase strongly when processing times are long. In cleaning efficiency tests the results showed that there are differences between detergents. In these trials the best cleaning result was achieved using 2-phase cleaning with 0.7% NaOH containing 0.2% SU 560 and 1.0% HNO_3. The 2-phase cleaning procedure using an alkaline mixture containing a chelator as well as HNO_3 was the most efficient combination for cleaning burned milk from stainless steel. In general, the results showed that the acidic treatment enhanced the cleaning result. The harmful thermophilic bacteria did not survive the cleaning treatments, but the remaining soil was also a risk factor offering an attachment site for new contaminants. The detachment of burned yoghurt-milk is therefore a very important but difficult task to achieve without strong mechanical forces. Some results are presented in the poster presentation Effects of cleaners of biofouled stainless-steel surfaces in yoghurt manufacturing equipment in Appendix 11.

2.5.3 Ozone compared with chemicals in milk CIP cleaning

The efficiency of cleaning and disinfection was evaluated using 3 different CIP procedures, which included a standard alkaline/acid wash, an enzyme-based treatment as well as ozonated water (see Chapter 5.8). Two strains of *B. cereus* were used in the 2 soiling procedures. The evaluation was performed measuring the number of germinating spores, i.e the disinfection effect, and the amount of remaining milk-soil, i.e. the cleaning effect.

Figure 5. Pilot equipment in simulation of soiling surfaces with burned yoghurt-milk.

Figure 6. Pilot equipment in soiling surfaces with heated yoghurt-milk.

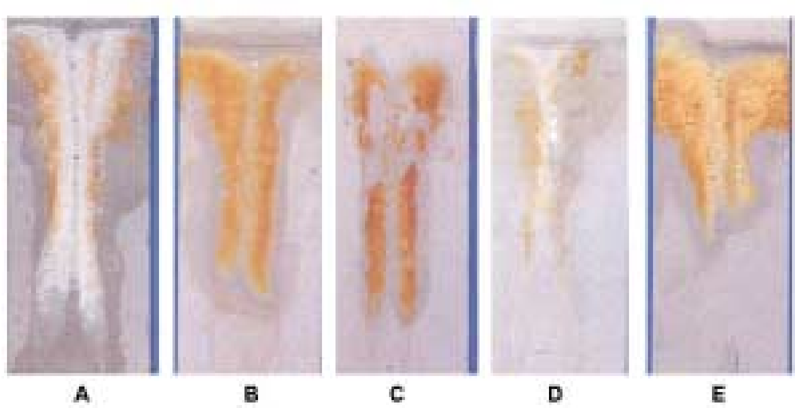


Figure 7. Stainless-steel surfaces soiled with burned yoghurt-milk containing harmful thermophilic bacteria: a) before cleaning, b) after single-phase cleaning with 0.7% NaOH, c) after 2-phase cleaning with 0.7% NaOH and 1.0% HNO_3, d) after 2-phase cleaning with 0.7% NaOH containing the chelator solution and 1.0% HNO_3 and e) after single-phase cleaning with 0.7% NaOH containing the chelator solution.

The sprayed spores displayed higher total cell numbers and smaller standard deviations, compared with the naturally adhered spores. The logarithmic reduction (4–6 units) of colony-forming units(CFU) on the steel surface after the different CIP procedures measured with the swabbing clearly showed that the standard wash with alkaline and acid was the most effective washing procedure. With the enzyme-based agent no significant reduction in cell numbers was observed. The ozonated water displayed reductions between 1–3 log-units. All of these measurements showed lower reductions for the naturally adhered spores in milk compared with the spores sprayed without milk. The TTC method worked very well for low contamination levels, but all reference surfaces were overgrown and a quantitative value could not be obtained. This method also showed that the standard washing procedure was the most effective one. Using the enzyme-based agent the surfaces were still overgrown after the cleaning, while with ozonated water the reduction was estimated to be 1–3 log-units.

Measurements of the remaining milk-soil were more difficult than measurements of the number of surviving spores, because all 3 CIP procedures evaluated are quite effective in removing milk-soil. It is difficult to find analytic methods sensitive enough for measuring the very small remaining fractions. Five different methods were tested and 4 gave poor results. The best results were achieved using the UV microscopy method, in which the area fraction of soil covering the surfaces was estimated. The most striking result was that spores without soil were shown to be extremely adhesive and almost impossible to remove with CIP procedures (see also Husmark, 1993). These results indicate that the standard wash was the most effective, followed by ozonated water and the enzyme-based agent. The contact angle method may be useful in the future, if the surfaces are measured directly after CIP and drying. The method is rapid, simple and sensitive; however, in this test the washed surfaces were rinsed in neutralizing solution before the contact angle was measured. Due to interference with the detergent present in the neutralizing solution, the results obtained were therefore not reliable. The SwabNCheck measured 4 units for the references and 0 for the standard wash and the wash with ozonated water. A slight change in colour could be registered from the enzyme-based agent. The ATP method gave more than 9000 relative light units (RLUs) from the reference surfaces and below 30 for all the other washed surfaces, which is equal to the background level. The macroscopic UV illumination gave a bright white-yellow shine on the reference surfaces but nothing could be seen after the cleaning procedures.

2.5.4 Potentiation of the disinfectant effect with alkali and enzyme wash

B. cereus present in pipes and heat exchangers represents a potential quality problem for the dairy industry. Disinfectants based on peroxygen can eliminate spores, but they are corrosive at high temperatures and concentrations (Russell, 1990; Bloomfield, 1992; Bloomfield & Arthur, 1994; Russell & Chopra, 1996). In the dairy industry, disinfection of pipes, tanks and pasteurizers is preceded by an alkali wash, often using temperatures above 60 °C, and alkali-treated spores are usually not used when testing the sporicidal effects of disinfectants. Contact with alkali could make the spores sensitive to peroxygen, reducing the concentration needed to obtain an acceptable killing level.

Resistance of spores to peroxygen

Blakistone *et al.* (1999) determined the lethal effect of Oxonia Aktiv (2%, 40 °C) on a number of spore-forming bacteria and showed that *B. cereus* spores were the most resistant. The resistance of *Bacillus* spores to disinfection varies with the strain, sporulation, harvesting and washing procedure, and storage conditions and recovery conditions (Waites & Bayliss, 1980). *B. cereus* ATCC 9139 spores were used in this study, because preliminary experiments revealed this strain to be the most resistant to Oxonia Aktiv compared with 8 *B. cereus* strains isolated from the Norwegian dairy industry (not shown). The higher resistance shown by of *B. cereus* ATCC strains than by dairy isolates to chlorine disinfectants was demonstrated by Te Giffel *et al.* (1995).

The sporicidal effect of disinfectants increases with temperature and exposure time (Bloomfield, 1992). A relatively high temperature (40 °C) and long exposure time (30 min) were required to obtain a significant reduction in *B. cereus* viable counts when applying the highest recommended in-use concentration of Oxonia Aktiv. Exposure to the recommended in-use concentration of another peroxygen-containing disinfectant, Parades (0.25%), was also not sporicidal for intact spores. The results suggested that the disinfectant must be used at either high concentrations or at high temperatures to be able to eliminate spores (Appendix 12). Apparently this is not suitable for practical disinfection due to corrosion and toxicity.

Potentiation of spores by peroxygen in alkali wash

The sporicidal effect of 1% Oxonia Aktiv was generally poor at 20 ^{o}C and 30 ^{o}C, even after an exposure time of 30 min. The sporicidal effect increased with higher temperature and exposure time; a log-reduction of more than 2 log-units was obtained after 30 min at 40 ^{o}C. A concentration of 0.2 % Oxonia Aktiv had little effect (< 1 log-unit) on the spores even at 40 ^{o} C and 30 min. Exposure to 1% NaOH (10–30 min, 60 °C) did not reduce the viability of *B. cereus* significantly (< 0.2 log-unit reduction). However, pretreatment of spores with 1% NaOH at 60 ^{o}C made the spores susceptible to Oxonia Aktiv, even when this was applied at a relatively low concentration (0.2%). The lethal effects of 0.2% and 1% Oxonia Aktiv were similar, indicating that a subpopulation of the spores was potentiated by the pretreatment, whereas the reminder of the population was unaffected even after 30 min of alkali treatment. Nevertheless, it appears that if contact between spores and warm alkali can be established, significant reduction in spore numbers can be expected even when using a relatively low concentration of Oxonia Aktiv.

The potentiating effect of NaOH occurred relatively rapidly within 10 min at 60 ^{o}C. Pre-exposure to 0.25% and 0.5% NaOH (60 °C, 20 min) affected the lethal activity of sAktiv, but much less than 1% NaOH. The effect of Oxonia Aktiv occurred relatively rapidly after alkali treatment, with maximum effect obtained within 10 min (Appendix 12).

The influence of cleaning temperature on the potentiating effect of alkali was investigated further. The effect was temperature-dependent and visible already at 40 ^{o}C. The alkali treatment was sporicidal at 80 ^{o}C and the additive effect of peroxygen disinfection was small at this temperature (Appendix 12).

The effect of Oxonia Aktiv on alkali-treated spores (1%, 60 °C, 20 min) was approximately 3 log-unit reduction (Appendix 12), which was equal to the exposure of intact spores to 1% HNO_3 (65 ^{o}C, 10 min). Alkali-treated spores were killed, showing > 5-log-units reduction by warm HNO_3 (65 ^{o}C, 10 min).

Potentiation of spores by peroxygen in enzyme-based treatment

Pre-exposure to the enzyme-based cleaner Paradigm (0.09%) at 60 °C for 20 min potentiated the effect of a peroxygen-based disinfectant Parades, but the effect

was generally higher when using 1% alkali at 60 °C for 20 min. A 3 log-unit reduction was obtained using the recommended in-use concentration of Parades, if the spores were pre-exposed to 1% NaOH. No lethal effect was observed if the pre-exposure was carried out using 60 °C water for 20 min (Appendix 12).

2.6 ENZYMATIC CLEANING

It is known that monocomponent enzymes can be used for biofilm removal. The heterogenicity of the biofilm matrix limits the potential of these enzymes for use in effective cleaning. The proteinase samples, e.g. chemotrypsin were shown to be effective in reducing and inactivating pure-culture biofilms, but when milk residues were present no effect of the proteinases could be observed. The different enzymatic cleaning procedures tested were also shown to be ineffective in inhibiting growth and metabolic activities of bacterial strains isolated from dairies. Based on the varying results obtained for removal and inactivation of microbes on surfaces by enzyme preparates, one possibility could therefore be to combine various types of enzymes to attain efficient cleaning. The use of enzymes is also limited due to the lack of techniques for quantitative evaluation of the enzymatic effects and the accessibility of the different enzymatic activities. The results showed that the resazurin-based fluorometric assay tested during that part of the project performed at the Faculty of Veterinary Medicine at the University of Helsinki can be used for estimating the enzymatic activities on process surfaces. This method can be recommended especially when a rapid, high-throughput capacity system is needed (Mikkola, 1999; Augustin, 2000).

2.7 TESTING OF DISINFECTANT EFFICACY

Efficient disinfection is required in food plants where wet surfaces provide favourable conditions for microbial growth (Bloomfield, 1988; Brackett, 1992; Holah, 1992). Disinfectants used in the food-processing industry include oxidizing agents, e.g. hypochlorite, hydrogen peroxide, ozone and peracetic acid; denaturating agents, e.g. alcohol-based products; nonoxidizing and surface tension diminishing agents; and enzyme-based compounds (Flemming, 1991; Troller, 1993). Although disinfectants were developed to destroy microbes (Brown & Gilbert, 1993), microbes have been found in disinfectant solutions. *Pseudomonas* sp. have been found in concentrated iodine solutions. As early as 1967 Burdon and

Whitby reported chlorhexidine ($C_{22}H_{30}Cl_2N_{10}$) mixtures contaminated with *Pseudomonas* sp. (Marrie & Costerton, 1981). Increased amounts of free chlorine (2.0 mg/l) did not kill *E. coli* grown in biofilm. The capsular *Klebsiella pneumoniae* has been shown to have a 150-fold resistance to chlorine when growing on glass surfaces compared with suspensions. Microbial contamination has also been found in solutions of aldehydes, quaternary compounds and amphotensides (Heinzel, 1988).

Determination of disinfectant efficiency is often performed in suspension tests with ready-to-use dilutions. Such tests do not imitate the growth conditions on surfaces, where agents are required to deactivate the microbes (Wirtanen, 1995). Various surface tests have shown that surface-attached cells are more resistant to disinfectant treatment than are cells in suspension (Mattila *et al.,* 1990; Wirtanen *et al.,* 1997). Results obtained using only one assessment method in testing can be inaccurate. For example, cultivation and staining with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and 4,6-diamino-2-phenylindole (DAPI) in a comparison based on biofilms showed underestimation of viable bacterial counts in the cultivation (Wirtanen *et al.,*1997). Testing procedures, based on cultivation, image analysis, impedance and metabolic indicators, e.g. CTC-DAPI staining, appear to give good estimation of both removal of biofilm from surfaces and killing of bacteria on surfaces. These methods assess different parameters and therefore complement each other (Bredholt *et al.,* 1999). A major problem associated with the testing of disinfectants against biofilm-grown bacteria is that it is difficult to recover all the surviving biofilm bacteria as single-celled CFUs, because sampling in surface tests is often based on cultivation of swabs taken from the test surfaces. Wood *et al.* (1996) and Gilbert *et al.* (1998) describe the use of poloxamer hydrogels for the construction of model biofilms in which the cell population expresses a biofilm phenotype and is present locally at high cell density, and in which resistance to disinfectants has increased substantially. Methods for disinfectant testing, however, require further validation.

2.7.1 Suspension tests

Bactericidal suspension tests are often used for determining resistance in bacteria, due to limitations in the minimum inhibitory concentration (MIC) method. In the simplest version of the method, bacteria are inoculated in disinfectant diluted in distilled water and the number of survivors after a

specified time and temperature, e.g. at 20 °C for 5 min, is determined by plate spreading. The main advantages of these methods are that all types of disinfectants can be tested and the effects of temperature, exposure time and interfering substances may be included. Bactericidal tests are easy to perform, but more time-consuming and less reproducible than the MIC test (Nicoletti *et al*., 1993). For this reason bactericidal tests are mostly used when investigating a few strains and a small number of antibacterial agents.

It is important to understand that total kill in a suspension will not guarantee efficacy in practical use. In most cases, bactericidal suspension tests for determining the efficacy of disinfectants will overestimate the effect in practical use. If a microbial strain survives the recommended in-use concentration at the recommended exposure time and temperature, it would most probably resist disinfection in practical use.

2.7.2 Surface test based on biofilms

The 1-N-phenyl-naphthylamine (NPN) test revealed that the tenside-based and peroxide-based disinfectants were effective permeabilizers of the *Pseudomonas* test strains used, whereas the alcohol-based agent was shown to permeabilize the test strains only weakly. In the biofilm experiments, the assessment based on CTC-DAPI staining showed that treatment with an undiluted alcohol-based disinfectant left more than 100 000 viable cells/cm^2 of both *Pseudomonas* strains on the stainless-steel surfaces. The reduction in viable cell counts was less than 1.8 log-units after treatment. Cultivation and impedance measurement showed that a 30-min treatment with the alcohol-based disinfectant was needed to reduce the amount of contaminants efficiently. In impedimetric assessment, bacterial growth was detected following an incubation period of more than 30 h after the biofilm samples were treated for 30 min with the disinfectant. The alcohol-based disinfectant proved to be very effective in experiments using *Pseudomonas* biofilms, when evaluated using impedance and conventional cultivation but not with CTC-DAPI staining, showing that many respiring cells were left on the surface after treatment. This shows that the alcohol-based agent deactivates the bacterial cells without destroying them (Larson & Morton, 1991). This was supported by the findings from permeabilisation using the NPN uptake assay, which showed that this agent affected the outer membrane (OM) only to a small extent. This effect could also be seen from the results based on a biofilm-construct of

hydrogel, which protects the cells (Wirtanen *et al.,* 1998). Denaturation can be very rapid in suspensions (Härkönen *et al.,* 1999); however, alcohols are not efficient against spores, which means that they do not destroy enzymes taking part in spore formation (Larson & Morton, 1991).

All methods used showed that the tenside-based disinfectant treatment left a large number of viable cells on the surface. The area covered by biofilm remained as it was before this disinfectant treatment. The tenside-based disinfectant was not effective against biofilm bacteria both in these experiments and in the hydrogel-construct test (Wirtanen *et al.,* 1998; Härkönen *et al.,* 1999). These findings are in agreement with those from a disinfectant efficiency study using dried bacterial cells on stainless-steel surfaces, in which a cationic tenside-based agent also proved to be ineffective in killing bacteria (Grönholm *et al.,* 1999) and in results achieved using *P. fragi* biofilm (Wirtanen, 1995). Cultivation and microscopy of CTC-DAPI stained biofilm on stainless-steel surfaces confirmed that the tenside-based agent was not effective on cells in biofilms. Impedance measurement, however, showed that the disinfectant was effective in solution. This effect could also be seen from the permeabilization results and from suspension tests (Wirtanen *et al.,* 1998), which suggests that this tenside-based agent affects naked cells but not cells embedded in biofilms or attached to surfaces.

Treatment using the peroxide-based disinfectant lowered the number of respiring cells. The cultivation results showed no growth in the treated *P. fragi* samples, whereas the number of cells in treated *P. aeruginosa* samples decreased after 10 min of treatment by 2.3 log-units and after 30 min of treatment by 5.0 log-units. The impedimetric assessment showed that some unharmed cells must have remained on the surfaces because the detection times were not longer for treated samples than for the controls. Some unharmed cells can grow very rapidly, resulting in very short detection times. In treatment with peroxide-based agents, the surface-attached cells burst in the treatment, which led to an increase in the area covered by debris compared with untreated samples. The microbicidal effect of peracetic acid on microbes in biofilms has been found to be limited, which means that the biofilm must be broken in some way before it can be used effectively (Exner *et al.,* 1987). The effect of hydrogen peroxide is based on the production of free radicals that affect the polysaccharides and glycoproteins in the biofilm. It is also known that hydrogen peroxide was effective in removing biofilms from surfaces (Mattila-Sandholm & Wirtanen, 1992). This effect was also noted in the

hydrogel test (Wirtanen *et al.,* 1998). The NPN uptake assay showed that the oxidizing peroxide-based disinfectant permeabilized the cell envelopes of the *Pseudomonas* strains used. This study proved that the peroxide-based disinfectant was the most effective disinfectant against *Pseudomonas* biofilms when the microbiological activity was measured using conventional cultivation and DEM with CTC-DAPI after a 30-min treatment. The impedance measurement showed, however, that some viable cells were left on the surface. Some unharmed cells can grow very rapidly, resulting in very short detection times (Wirtanen *et al.,* 1997).

Treatment with the chlorine-based disinfectant lowered the amount of respiring cells below the detection level, as did the peracetic acid treatment. The range of microbes killed or inhibited by chlorine-based compounds is probably broader than that for any other approved agent (Holah, 1992; Tuncan, 1993; Helander *et al.,* 1997). The cultivation results showed no growth in the treated samples when the duration of the disinfection was prolonged to 30 min. After 10 min of treatment the counts were lowered by 3.1 log-units in *P. fragi* samples; in *P. aeruginosa* samples no growth was found. According to the impedance results no growth in the samples was found within a 24-h period after a 10-min treatment. The cultivation and impedance measurement showed that prolonged treatment with the chlorine-based disinfectant was especially efficient on *Pseudomonas* biofilms. The antibacterially active moiety is formed when chlorine or hypochlorite compounds are added to water and hypochlorous acid (HClO) is produced. Stabilized hypochlorites are used when disinfection of long duration is required (Troller, 1993). The results in the NPN uptake assay showed that the cell envelope was not harmed. The results from the hydrogel test (Wirtanen *et al.,* 1998) were in agreement with these findings and showed that the chlorine-based disinfectant did not penetrate the thick layers of biofilm constructs e.g. hydrogels. Findings, especially from the impedance measurement showed that only small changes occurred in the cell envelope. Dychdala (1991) suggested that the bactericidal effect of hypochlorites is based on penetration of the chemical into the cell as well as chemical reaction of the chemical with the cell protoplasm, i.e. oxidative action on essential enzyme systems in the cell.

The influence of fluid dynamic shear of disinfectant solutions on the detachment and killing of *P. aeruginosa* in biofilms formed on the stainless-steel surface in a CCR reactor was also studied. The results showed that the CCR can be used to

discriminate between biocidal and cleansing action for different disinfectant types (Appendix 13).

2.7.3 Surface test based on biofilm constructs

The results show that the Gram-positive bacteria tested in poloxamer hydrogels underwent killing that varied in extent from ~ 0.1 to ~ 2-log-unit reductions. The least susceptible organisms were *M. luteus* E-215 and *L. monocytogenes.* The most effective agent against these 2 bacteria was a peroxide-based disinfectant HPPA-1. This treatment was the poorest against *L. innocua*. It was also the most effective of the formulations against the *Salmonella* strains tested, showing a reduction of approximately 1 log-unit. The isopropyl alcohol-based IPA-L was effective against most of the tested bacteria, except *E. coli* and *L. monocytogenes*. The tenside-based disinfectant showed poor efficacy with all the microbes tested. In the suspension tests using 4 the above-mentioned disinfectants against the 11 bacteria, a 5-log-unit reduction in viable count (5 min) was achieved in all instances, in many cases with no recoverable viable cells. The susceptibility of the poloxamer gel constructs to HPPA, was further evaluated over a range of concentrations, representing the extremes and mid-point of the recommended use levels, using *P. aeruginosa*, *E. coli, L. innocua*, *B. subtilis* and *S. epidermidis*. In most instances the degree of effectiveness increased with increasing exposure to the agent. The results in the second study, in which various commercial formulations of the same type were evaluated against biofilm constructs inoculated with *P. fragi, Enterobacter* sp., *L. monocytogenes, B. subtilis* and *Dekkera anomala*, confirmed the earlier results, showing that there is a pattern of susceptibility varying as a function both of the organism and the disinfectant type. The results gainedfrom testing 13 commercial disinfectants showed agreement with the general observation that Gram-negative bacteria are more resistant to disinfectant treatments than Gram-positive bacteria (Vaara, 1992; McKane & Kandel, 1996). In all the gels with Gram-negative bacteria, significant levels of surviving bacteria were detected. The most effective formulations in these tests were the oxidizing hydrogen peroxide based disinfectants. However the activity of this type of agent, unlike the other formulations, was much lower against *L. innocua* and *L. monocytogenes*. The killing activity generally increased with greater exposure to the agent. They also performed best against Gram-negatives, e.g. *Enterobacter* spp., *Salmonella* spp. and *P. fragi* as well as against the Gram-

positives *L. monocytogenes* and *B. subtilis* (vegetative cells). The isopropyl (IPAs) and ethyl (ETA-B) alcohol-based disinfectants proved to be more effective against vegetative cells of the Gram-positive *B. subtilis* than against the other microbes tested (Appendix 14). The tenside-based disinfectant TAAS was also the least effective in the hydrogel tests against all the microbes chosen (*P. fragi, Enterobacter* spp., *L. monocytogenes, B. subtilis,* and *D. anomala*), giving a log kill of < 0.3. These results agree with earlier studies using bacterial cells dried on stainless steel surfaces as the inocula (Grönholm *et al.,* 1999) and with biofilm studies (Wirtanen *et al.,* 1998, 2001).

The test based on hydrogel constructs is a severe test of disinfection efficiency. Whilst the results do not necessarily reflect the likely effects of a formulation against microbial contamination *in situ,* they do discriminate between the disinfectant formulations used at normal levels. The differences in activity between the two *Listeria* strains highlight the need to use realistic problem organisms, such as *Salmonella* spp. and *L. monocytogenes,* in testing programmes. Conventional suspension tests fail to discriminate between the agents in terms of their efficacy and would therefore not assist in the selection of agents (Appendix 14).

3. MICROBIAL PHENOMENA

3.1 RESISTANCE PHENOMENON DUE TO USE OF DISINFECTANTS

3.1.1 Definition of resistance

For users of disinfectants in the food industry and in other applications, it would be most relevant to define resistance as 'survival in practical use'. However, survival after disinfection may be explained by factors not related to the properties of the microbes themselves, but to external factors, such as soil neutralizing the disinfectant. Survival may also be explained by other failures in the cleaning procedures, e.g. leaving too much water after cleaning which will dilute the disinfectant to sub-lethal levels or using temperatures too low or exposure times too short during disinfection.

In this study, bacteria surviving the lowest recommended in-use concentration of a disinfectant in a simple bactericidal suspension test are termed resistant. Since many factors affect resistance in practice, a low-level resistance not detected in this test may contribute to survival in the dairy plant. Therefore, bacteria not surviving this test may have survived practical disinfection due to a combination of resistance and biofilm formation.

3.1.2 Resistant strains from dairies

The results of hygiene tests after fogging disinfection and of disinfecting footbaths is described in Chapters 2.3 and 2.2 respectively. Procedures for isolation of resistant bacteria are presented in Chapter 5.1.

Identification and characterization of bacteria isolated from disinfecting footbaths and fogging showed that some strains were resistant to the disinfectants applied. The microbial flora in disinfecting footbaths with TEGO and alkylamine-based TP-99 was dominated by Gram-negative bacteria (coliforms and pseudomonads) and spore-formers. In baths with oxidative disinfectants the flora consisted mainly of Gram-positive bacteria, *Acinetobacter*-like microbes and spore-formers. Strains isolated from used chlorine compounds, TEGO, TP-99 or Oxonia Aktiv, or from the surface of footbaths with the respective compounds, were exposed to the recommended in-

use concentrations of disinfectants based on chlorine, amphotheric tenside, alkylamine, quaternary ammonium compounds or peroxygen in a bactericidal suspension test. Possible cross-resistance was also studied. Spore-forming strains were not tested because they are intrinsically resistant to most disinfectants. In general, bacteria from the footbaths or from the disinfectants used did not survive exposure to the recommended in-use concentrations of chlorine, alkylamine or peroxygen. Resistance to oxidative disinfectants has mainly been associated with biofilm growth (Bolton *et al.,* 1988; Clark *et al.,* 1994; Mead & Adams, 1986). In essence, this indicated that survival in footbaths containing hypochlorite and Oxonia Aktiv was not mainly caused by development of high resistance, but by biofilm formation.

Some bacteria isolated from footbaths using an amphoteric disinfectant TEGO or from used TEGO survived exposure to 1% TEGO or 200 μg/ml benzalkonium chloride ($C_6H_5CH_2(CH_3)_2NRCl$), and were considered resistant. A permeability assay based on NPN showed that resistance was probably due to low membrane permeability.

In the investigation of fogging disinfection, disinfectant resistance was tested for some strains surviving in apparently clean areas. In many dairies, the microbial flora after disinfection was dominated by a Gram-negative, slow-growing microbe forming red colonies, which was identified as *Methylobacterium* sp. by fatty acid analysis. These bacteria were not resistant to the alkylamine-based TP-99 disinfectant applied when grown in rich laboratory media prior to exposure in the bactericidal suspension test. However, after starvation in 10% nutrient medium, they survived in the bactericidal test. These bacteria would not have been detected in the ordinary hygiene testing performed in the dairy, because visible colonies appeared only after 5–6 d incubation. In one dairy a Gram-positive bacterium resistant to several disinfectants was isolated; this strain was identified as either *Rhodococcus* sp. or *Nocardia* sp. by fatty acid analysis.

3.2 Thermophilic Bacteria in Yoghurt Processing

The causes for delays in yoghurt fermentation were studied by isolating harmful thermophilic bacteria from the yoghurt process. Bacterial numbers were determined from hot- and cold-mixing equipment used in yoghurt manufacturing. Milk samples were taken from the evaporator funnel, sampling valve in the pasteurizing apparatus

and the fermentation tanks. Milk samples were taken 0, 2, 4, 5, 6, 7, 8, 9 and 10 h after the beginning of processing. Milk samples were cultivated on milk plate count agar and the plates were incubated at 55 °C for 48 h. The results showed that the numbers of thermophilic bacteria were lower when using the cold-mixing than the warm-mixing method. The numbers of thermopholic bacteria increased significantly after an 8-h production.

Eight isolated thermophilic bacteria were identified. Gram-staining, spore-staining, catalase test, API and ID tests as well as ripotyping were performed. All identified strains were Gram-positive, spore-forming rods belonging to the genus *Bacillus* (*B. flavothermus, B. licheniformis* and *B. subtilis*).

Effects of 2 different spoilage bacteria in yoghurt fermentation were tested on a laboratory scale. Bacteria were added into ultrahigh-temperature (UHT) milk together with normal starter cultures. The duration of yoghurt fermentation was determined by measuring the pH as a function of time; the yoghurt was ready when the pH was < 4.5. Fermentation tests showed clearly that the thermophilic bacteria isolated from the process prolonged the yoghurt fermentation. *B. licheniformis* prolonged the fermentation process more than *B. flavothermus*. *B. licheniformis* numbers of > 8.0×10^5 cfu/g prolonged fermentation up to 1 h.

3.3 *BACILLUS* SPORES ON PROCESS SURFACES – INVENTORY STUDY OF TOTAL CONTAMINATION AND *B. CEREUS* ALONG A CREAM PRODUCTION LINE

B. cereus is an ubiguitous organism readily adhering to surfaces of production equipment. *B. cereus* spores appear to be relevant indicator organisms for measuring cleaning effects. This study was carried out to increase our knowledge of the frequency and localisation of contamination with *B. cereus* in closed equipment. The work also aimed at improving methods for measuring the cleaning effect. The 2 methods used in this case, alginate swabs and moulding technique, functioned both quite well. Improvements can be made concerning how to solve the alginate swabs satisfactorily and whether a detergent can be used in the swabbing for better recovery. The moulding technique can probably be made more sensitive with some further improvements in the substrate used. The methods for quantification of growth on moulded parts can also be improved. The results from this study showed that several sites had quite high

levels of contamination. Some sealings and gaskets, especially sealings in the pumps located before the filling stage, were heavily contaminated with Gram-positive rods and cocci. Also Gram-negative bacteria were found at some sites. There were 7 sites at which *B. cereus* was found both in the spore and in the vegetative form.

4. ENVIRONMENTAL ASSESSMENT

4.1 LIFE-CYCLE ANALYSIS FOR ASSESSING ENVIRONMENTAL EFFECTS OF CLEANING

The intention of this project was to assess environmental impacts of different CIP methods. The environmental effects of cleaning agents are usually judged on the chemical compound, and the energy and water consumption in the user phase. In this LCA study, in which a method for analysing and assessing the environmental impacts of a material, product or service throughout its entire life cycle was used, TINE cooperated together with chemical supplier. The different methods for CIP cleaning compared were conventional alkaline/acid cleaning (HNO_3 and NaOH) with hot-water disinfection, single-phase alkaline cleaning with acid chemical disinfection (Oxonia Aktiv S), enzyme-based cleaning (Paradigm) with acid chemical disinfection (Parades) and conventional alkaline/acid cleaning and disinfection with cold HNO_3 at pH 2.

In this LCA 3 different transport cases (Oslo, Stavanger and Alta) and 3 different effluent treatment methods (no treatment, internal biological effluent treatment plant at the dairy and municipal effluent treatment plant and in addition a method with filtration of CIP solutions) were used. The functional unit is defined as: *Satisfactorily cleaning, based on experience, of an average Norwegian dairy with 30 cleaning operations a day (excepting pasteurizers) through one year.* The required amount of energy, water and cleaning agents was based on earlier measurements and practical experiences. The chemical oxygen demand (COD), phosphorus (P) and nitrogen (N) emissions were calculated from the content of these elements in the cleaning agents. The production data of the cleaning agents was site-specific data from the producers.

Based on the assumptions and limitations of this LCA the method of enzyme-based cleaning has the lowest environmental impact. It has the best result for the evaluation methods EPS and Ecoindicator, and also for the impact categories global warming potential and ozone formation. The enzyme method and the single-phase alkaline method had almost the same and lower use of energy than the other methods, and also lower acidification potential. The enzyme-based cleaning agent is used in very small concentrations and at low temperatures, which are the main reasons for these results. The single-phase alkaline method showed the best result for eutrophication.

Transport did not greatly influence the results. Filtration of CIP solutions directly impacts the produced and transported amounts of cleaning agents, which leads to better results for all parameters considered. The other effluent treatment method, cleaning of wastewater, influences only the potential for eutrophication and the Ecoscarcity evaluation method. Cleaning of the dairy and production of cleaning agents and their delivery, have the greatest influences on the results.

Several sources of error occur in this analysis. The origin of the data is not the same for all the CIP methods, and many assumptions have been used. Emissions of various chemicals occur that cannot be evaluated or characterized in an LCA e.g. phosphonates and different tensides, which are part of some of the cleaning agents (the disinfectants of the enzyme method and the single-phase alkaline method) used in this analysis.

4.2 Evaluation Procedure for Functionality of Sanitation

The procedure used to evaluate sanitation functionality applies to both new products and products already inuse (Fig. 8). The following sequences should be included:

a. Overlapping assessment of cleaning/disinfecting

The first estimate of suitability for use is made based on fundamental theoretical aspects:

- The product content from the specification sheet
- Environmental influence should be assessed
- Theoretical assessment of functional properties based on data from the supplier's tests performed on a small scale. The criteria for checking include the influence on different types of goods, ability to remove and transfer soil, killing effect on microbes as well as technical properties (foaming properties, stability etc.)
- A cost estimate.

b. Supplier audit

- The cleaning and disinfection agents should be checked based on answers to process-specific questions.

- Delivery certainty, product safety, responsibility of the producer, quality aspects as well as company economy should be assessed.

c. Assess the functional properties of cleaning/disinfection

Small-scale tests should be conducted to determine if the agent fulfils the demands set by the customer and the supplier. Suitable model tests e.g. the cheese mould test, surface test for open surfaces and CIP tests should be conducted on a large scale. The criteria should include: the influence e.g. corrosion and deterioration of the goods used, ability to remove and transfer the soil, rinsabilty, killing effect on microbes, technical properties e.g. foaming and stability as well as the recommended concentration, temperature and duration.

d. Practical evaluation

New cleaning or disinfection agents should be checked after certain intervals during the first year. A performance plan should be in effect, including above criteria. The methods used for cleanliness should include visual inspection and measurement with UV and ATP of critical points, concentration, duration of cleaning or disinfection, flow using nozzles as well as soiling in the CIP tank and phase differentiation.

e. Final decision

The environmental impact, functional properties as well as the economy should be used in the final decision (Fig. 8).

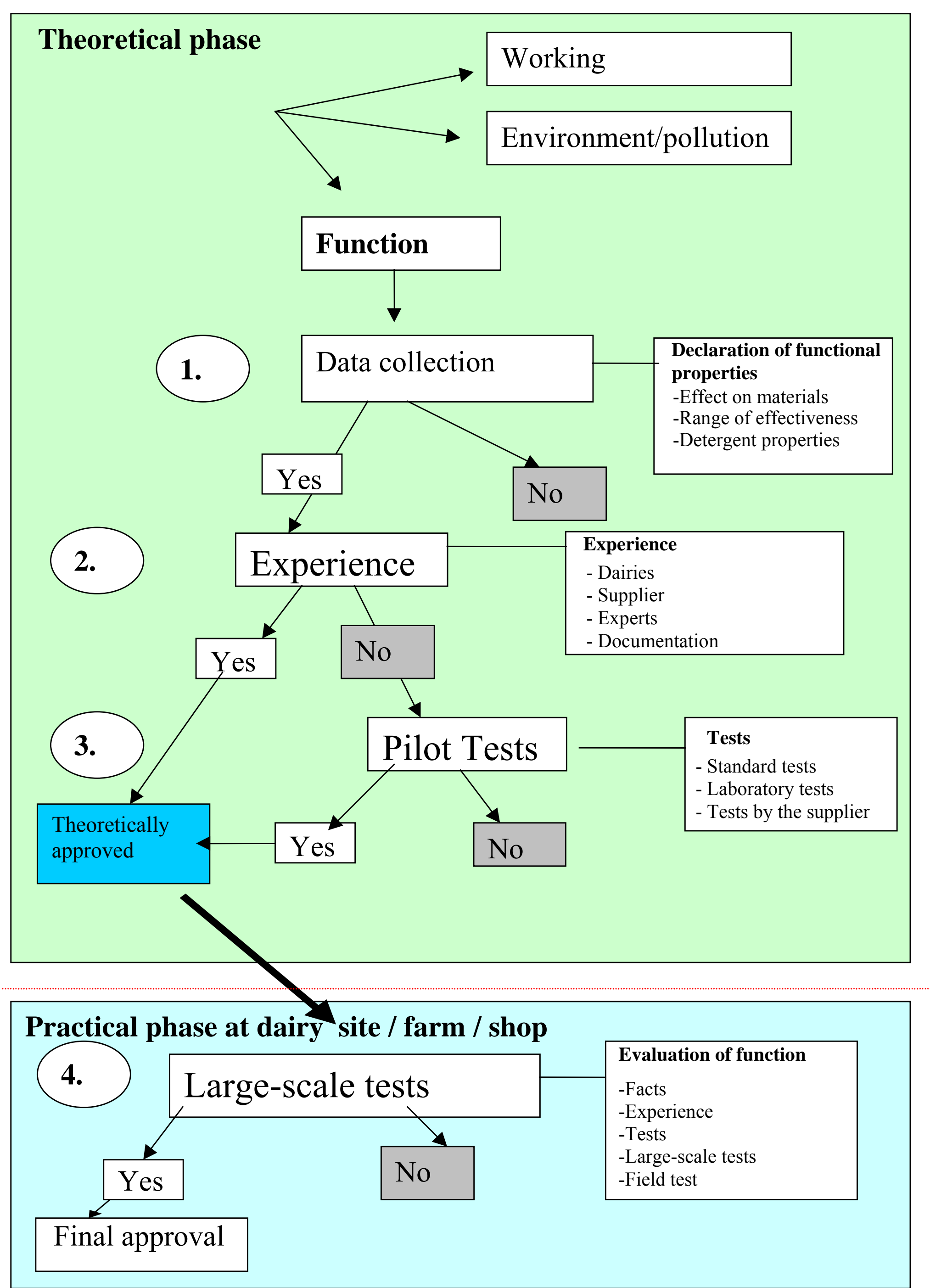


Figure 8. Evaluation procedure of the sanitation processes.

5. DETECTION METHODS USET IN THE STUDIES

5.1 ISOLATION OF RESISTANT MICROBES

5.1.1 Commonly used methods for testing resistance phenomena

The MIC method has been used extensively to determine resistance to antibiotics and is also used for disinfectants. In this method, one or several bacterial strains are inoculated in a range of concentrations of disinfectant in nutrient broth (NB). The lowest concentration allowing bacterial growth after a specified time, e.g. 24 h, is termed the MIC-value of the disinfectant for the microbe. The main advantages of the MIC method are that it is easy to perform and many strains or antibacterial agents can be tested in the same experiment. The applicability of the MIC method is limited because many commonly used disinfectants cannot be tested because their pH is either too high or low for growth or precipitation of the disinfectant in the NB (Nicoletti *et al.,* 1993; Sundheim & Langsrud, 1995). The relevance of the method has also been questioned since the aim of disinfection is not primarily to prevent growth, but to kill microbes. Therefore, the MIC method is most commonly used in screening of strains for resistance, comparing the efficacy of antibacterial agents or studying synergy effects.

It has been demonstrated that bacteria isolated from disinfectant solutions or disinfection equipment can lose their resistance rapidly under laboratory conditions and may not survive exposure to the disinfectants in laboratory tests (Lowbury, 1951; Carson *et al.,* 1972; Heinzel & Bellinger, 1982). One explanation for this phenomenon is that under natural conditions the microbes grow on surfaces whereas they have been cultivated in nutrient broth prior to exposure to the disinfectant in the laboratory tests. It is now well known that bacteria attached to surfaces are generally more resistant to a range of antibacterial agents, including disinfectants (LeChevallier *et al.,* 1988). Following attachment, bacteria produce extracellular polymers protecting them from the disinfectant applied. Other factors may also contribute to the resistance of cells in natural environments such as growth rate reduction, limited nutrient availability and increased production of biocide-degrading enzymes (Brown & Gilbert, 1993; Boulange-Peterman, 1996). Thus, the survival of microbes under practical conditions is not only a result of their resistance, but also of their ability to attach to surfaces and their state. Bactericidal tests on bacteria attached to surfaces may be used to determine resistance of microbes. These tests may give

more realistic views of the levels of disinfectant that will kill the microbe in practical surface disinfection in factories with low hygienic level. In practice, surfaces are cleaned before disinfection and the exposure to cleaning agents may reduce the resistance of the microbes to disinfectant (Holah *et al.,* 1998; Langsrud *et al.,* 2000).

5.1.2 Methods for isolating resistant strains

When bacteria survive cleaning and disinfection at unacceptable levels in the food industry this can in most cases be explained by weaknesses in cleaning procedures and practical performance. However, sometimes the survival is due to high resistance to disinfectant. The following procedure was used in this study to determine if survival was due to high resistance:

Potential resistant isolates were identified. It is well known that spores and mycobacteria survive high levels of disinfectants and identification could be sufficient to explain survival in many cases. For certain types of bacteria intrinsic resistance to some disinfectants and biofilm formation have been reported, e.g. pseudomonads, and this could give some indications of why they survive practical disinfection levels.

If it was not evident from the literature that the bacteria have high intrinsic resistance to the disinfectant applied, we tested the level of resistance. The strains were analysed in a simple suspension test in distilled water using the recommended in-use concentration of the actual disinfectant. In some cases we tested type strains or reference strains from other sources of the same species. If type strains and reference strains were susceptible in the suspension test, this indicated that the species generally was not expected to survive disinfection. If they survived the test, the recommended in-use concentration of the disinfectant may have been too low to kill the actual species. If the isolate survived the suspension test it was termed resistant. If it was killed, we did not know if it was susceptible or had low-level resistance.

If the isolate survived the suspension test it was necessary to determine if it had survived or grew in the disinfectant solution for a longer time, especially if it had been isolated from a disinfectant solution. If the isolate was killed by the suspension test, it was on some occasions tested to determine if other growth

conditions or disinfection procedures more similar to those in practical use made the isolate resistant. To find measures to eliminate a resistant isolate, potentiation of disinfectant by a cleaning agent and/or cross-resistance to other disinfectants was on some occasions evaluated.

5.2 DISINFECTANT TESTING USING BIOFILM CONSTRUCTS

The poloxamer hydrogels demonstrate thermoreversible gelation properties, being liquid and fully miscible with water at temperatures <15 °C but firm gels at temperatures >15 °C. This suggests that dense populations can be cultured and exposed to disinfectant as a gel at 30 °C, from which full recovery of individual cells can be achieved simply by placing the hydrogels in neutralizing solutions/diluents at <15 °C (Wirtanen *et al.,* 1998). The poloxamer Pluronic F127 has been investigated earlier for its potential as an agar substitute in microbiology. Solutions are unaffected by autoclaving and appear to be nontoxic to all bacterial species so far tested (Gilbert *et al.,* 1998; Wirtanen *et al.,* 1998; Härkönen *et al.,* 1999). The poloxamer matrices in the present study not only reproduce the reaction-diffusion resistance properties of the biofilms but also simulate other aspects of the biofilm mode of growth. The present paper evaluates the possible use of such biofilm constructs of process contaminants for comparison of commercial disinfectant formulations at normal use concentration.

Poloxamer F127 (Uniqema, Belgium) is a diblock copolymer of poly-oxyethylene and polyoxypropylene. Aqueous solutions show thermoreversible gelation. Poloxamer flakes were made up to 30% w/v in tryptone soy broth (TSB) and refrigerated overnight to allow hydration to occur. The dissolved poloxamer solutions were then autoclaved and returned to the refrigerator. Before use, sterile, chilled poloxamer (3 ml) was inoculated with a 1/100 dilution of overnight (16–18 h) cultures (300 µl) of e.g. pseudomonads, enterobacters, micrococci, bacilli, staphylococci, listeria, salmonella and yeasts in fresh TSB to form a biofilm culture. The drops of poloxamer were carefully placed onto stainless-steel (AISI 304, 2B) discs placed in Petri dishes containing a small piece of moistened cottonwool. Each Petri dish was sealed with Petrifilm and incubated at 30 °C in a static incubator. The susceptibility to in-use concentrations of various commercial disinfectants, e.g. based on hydrogen peroxide, hypochlorite, alcohols and tenside, was assessed after exposure for

5 min at 25 °C in the hydrogel test. After 5 min, samples (1 ml) were transferred to a neutralizer solution (9 ml) which was kept under refrigeration (10 ± 1 °C) for the hydrogel test and an ambient temperature (22 ± 1 °C) for the suspension test. The aliquots were left for 5–15 min before further dilution and viable counting on TSA plates (incubation 2–3 d at 30 °C) and yeast and malt extract agar plates (incubation 4–5 d at 25 °C). The suspension test was performed twice. The poloxamer-grown cultures were transferred directly to prewarmed solutions of disinfectant, together with the stainless-steel discs. These tests were carried out in triplicate. After 5 min the discs were removed and the gels transferred to solutions of neutralizer (10 °C) for 5 min. This was sufficient time for the gels to liquify and disperse. Serial dilutions were made and viable counts estimated as before. The results were expressed as survival relative to appropriate controls following exposure to sterile water (hydrogel test) or saline (suspension test). The neutralizer solution contained lecithin (0.6% w/v), Tween 80 (6% w/v), sodium thiosulphate (0.8% w/v), L-histidine hydrochloride (0.5% w/v), and bovine serum albumen (0.72% w/v) in Sorenson's phosphate buffer (1.25 mmol). The neutralizer solutions were sterilized by filtration through a 0.45-µm filter. The efficiency of the neutralizer had previously been tested using suspension cultures of *M. luteus* VTT-E-91474.

5.3 DISINFECTANT TESTING USING BIOFILMS FORMED ON SURFACES

The susceptibility of commercially available disinfectants at in-use concentrations was assessed after exposure for 10 and 30 min on 4-d-old *Pseudomonas* biofilms grown on stainless-steel coupons at 25 °C (60 ± 5 rpm) in slime broth (Wirtanen, 1995). The test coupons were removed from the biofilm formation vat and rinsed for 15 s in lukewarm (25 °C) sterile, distilled water in a small vat under mild stirring to release loose cells before the disinfectant treatments. This treatment was performed twice (Bredholt *et al.,* 1999). Three coupons of each type were transferred directly to disinfectant solutions (200 ml) at in-use concentrations (25 °C) for each of the 4 following measurements: cultivation, impedance, area coverage and metabolic indicators. After the treatments the coupons for cultivation were transferred to solutions of neutralizer (25 °C) for 5 min. The recipe of the neutralizer solution is given above (see Chapter 5.2). The aliquots were left for 5–15 min before further dilution and viable counting onto TSA plates (incubation: 2 d at 30 °C). Saline was used

instead of the disinfectant in the control test. The following determinations were performed:

- *Conventional cultivation:* The bacteria were scraped from the test surfaces (2.5 x 7 cm^2) with a cotton-tipped swab (see sample treatment in previous chapter), which was transferred into a test tube containing 5 ml maximal recovery diluent (MRD; LabM, UK). The test tube containing the swab was stirred thoroughly for 1 min to release the cells into the MRD solution. The samples were diluted as logarithmic series in MRD and cultivated at 30 °C on PCA for 3 d.
- *Impedance:* The change in impedance (resistance) of the growth medium due to microbial growth was automatically measured using a BacTrac 4100 instrument (Sy-Lab, Purckersdorf, Austria). The instrument consists of autoclavable glass measuring vessels containing 2 pairs of electrodes connected to a microprocessor. The metabolism caused by microbial growth 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, whereby weakly charged substrates in the growth medium are transformed into highly charged end products. Capacitance (the E-value; relative electrode impedance) can be altered by factors such as changes in the pH of the medium. The E-value should also be used if the growth medium contains a high concentration of salts, which may make 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 in the BacTrac incubator block (30 °C, 48 h), and detection time for the samples was measured when the sample reached the E-value set.
- *Epifluorescence image analysis of acridine orange-stained samples:* For the steel surfaces covered with biofilm stained with AO, the stain was allowed to act for 2 min at room temperature, after which the surfaces were rinsed with sterile water, air-dried and stored at 4 °C. The stained surfaces were examined under an Olympus AH-2 epifluorescence microscope (Olympus, Japan) with a suitable filter combination, using a 100x oil-immersion objective. Area measurements were carried out with a microcomputer system. The images (50 fields/sample) obtained in the microscope were analysed as grey scale interpretations. The areas covered with biofilm were converted into percentages of the total area analysed per coupon (Wirtanen, 1995).

- *Epifluorescence image analysis of samples stained in a metabolic indicator system:* CTC and DAPI staining was performed on biofilms attached to stainless steel surfaces. For direct staining of the coupons, 2 ml of 5 mmol CTC (Polysciences, Inc., USA) were pipetted onto the surface and incubated without shaking at 30 °C for 2 h. After incubation, the surfaces were rinsed with sterile distilled water, fixed in formaldehyde (CHCHO) solution, rinsed with sterile distilled water and air-dried at room temperature for about 20 min. A total of 2 ml of 1 µg/ml DAPI (Sigma, USA) was added to the surface. The stain was incubated for 20 min at room temperature, after which it was poured off. The coupons were rinsed with sterile distilled water and air-dried. The stained biofilm samples were stored at 4 °C. The samples were analysed using the BH-2 epifluorescence microscope (Olympus) witha total magnification of 1000x. The total number of cells and the number of living cells were counted from 15 microscopic fields (= 0.032 mm^2) for each sample. The results are given as actual number of cells per area; the detection limit in this experiment was 3000 cfu/cm^2. Image analysis was carried out with the Image-Pro Plus™ program (version 3.0 for Windows™; USA) using an Optronics OPDEI-470T Cooled Color charge-coupled device (CCD) camera (Optronics Engineering, USA) and a system consisting of a Targa 64+ ADC card image processor and a High Resolution SVPVM1353 13" RGB colour monitor image display (Sony, USA).

5.4 DISINFECTANT EFFICACY TESTING – CELL PERMEABILITY TESTING

The bacterial suspensions were treated in in-use concentrations except for the alcohol-based agent, of which the concentration was 0.5%, in 5 mmol N-[2-hydroxyethyl]piperazine-N'-[4-butanesuöphonic acid] (HEPES), pH 7.2 (Sigma). The effects of the disinfectants were assayed using the hydrophobic probe uptake method, in which a hydrophobic fluorescent NPN probe (Merck-Schuchardt, Germany) is added to the bacterial suspension and the resulting fluorescence is measured at 420 nm (excitation wavelenght 350 nm; Shimadzu RF-5000, Japan). An increase in fluorescence is associated with disruption of the OM of the bacterial cell, since NPN only fluoresces in a lipid environment. In an aqueous milieu the fluorescence is zero. Normally the OM prevents the entry of NPN into the cell's lipid layers; thus low fluorescence indicates the presence of an intact OM (Helander *et al.*, 1997; Helander & Mattila-Sandholm, 2000).

5.5 CONTROL METHOD FOR TESTING FOOTBATH HYGIENE

The sampling kit contained sterile test tubes, test tubes with NB, sterile swabs and pipettes. The managers were asked to take 4 samples (a–d) from 3 or 4 footbaths, preferentially from the most contaminated footbath, but with cleaner ones also included. We sampled about 50% of the footbaths in 11 dairies. The 4 samples were:

- Samples of the used disinfectant before emptying the footbath (3–5 ml in a sterile tube).
- One or two drops of disinfectant used added to sterile NB, which neutralizes the toxic effects of the disinfectant.
- Swab samples of about 10 cm^2 of a corner from the footbath after emptying. The swabs were broken aseptically into the tubes with NB.
- Swab sample of the footbath before refilling of disinfectant. Some plants washed the footbath before refilling.

A total of 10 µl of samples a–d were plated on PCA after arrival of the samples in the laboratory. The plates were incubated for 3–7 d at 20 °C. Colonies of differing morphology were further cultivated and stored in Lurcia-Berthani broth containing 15% glycerol at –80 °C.

5.6 METHOD FOR TESTING CHEESE MOULD CLEANABILITY

Contamination of cheese mould surfaces was carried out using autoclaved cheese slurry prepared from 50+ cheese. The bottom parts of the cheese forms were contaminated with the cheese slurry in combination either with lactic-acid bacteria from a starter culture for cheese or with *B. cereus* spores. The pieces of the moulds were immersed in the solution for 15 min and rinsed by dipping 5 times in clean water. After 1–2 h of drying the bottom parts were assembled and washed.

The cleaning equipment used in the pilot scale was a Jeros 5120 washing machine. Washing at 70 °C lasted 6 min with a water rinse for 1.5 min. The cleaning equipment used in the full-scale operation was a Tuchenhagen tunnel. The cleaning programme included prerinsing with water, cleaning and finally rinsing with water. The programme time was 3 min and the washing temperature

was 70 °C. After washing the level of microbial contamination was measured with different methods:

- In washing out (measures cleaning and killing effect) one piece of the mould was placed in a sterile bag containing sodium chloride (0.85%, 100 ml) and Tween (0.1%). The bag with the mould piece was shaken in a shaking device for 30 min. From this suspension the number of surviving bacteria was measured using the traditional pour plate technique.
- The TTC method measures the cleaning and killing effect. The cheese moulds with *B. cereus* spores or lactic-acid bacteria were moulded with TSA containing the indicator TTC. The cheese moulds covered with agar were incubated at 30 °C for 2 d. After this incubation the degree of red colouration was estimated manually (scale with 5 degrees) and measured with a colour camera. Ten measurements were taken of each mould piece.
- The ATP method was used to measure the cleaning effect. The special swabs were wetted in swabbing solution; the entire test piece (12 x 12 cm^2) was swabbed. The swab was immersed and shaken for 10 s in a releasing agent and the relative luminescence was measured in a luminometer.
- UV illumination can be used to measure the cleaning effect. A UV lamp was used for macroscopic visualization of contaminants. The cheese mould was illuminated with the lamp in a dark room.
- In the DEM method, which measures the cleaning and killing effect, the cheese moulds were incubated for 6 h in NB before staining with AO and analysis in a fluorescent microscope.
- Measurements of protein residues to measure the cleaning effect were performed using the SwabNCheck monitoring kit (LabDesign). The cheese mould was swabbed according to the test protocol. The colour change was visually inspected after 10 min and was also measured in a spectro-photometer at 562 nm. According to the technical information the sensitivity varied between 15 and 1000 μg protein.
- Swabbing, which measures the cleaning and killing effect, was carried out by shaking the swabs with which the surfaces were sampled in sodium chloride (0.85%) and the number of bacteria was measured through a conventional pour plate technique.
- Bioscreen measurements can be used to measure the cleaning and killing effect. The Bioscreen measures bacterial growth spectrophotometrically. This method was modified between the different trials. The cheese-mould was immersed either in various types of NBs or sodium chloride. From this

suspension assays of 0.1 ml were transferred to the Bioscreen for measurements lasting 24–45 h. The integrated area under the growth curves was calculated.

5.7 METHOD FOR TESTING AIR DISINFECTION EFFICACY

Contamination of the surfaces used in the fogging experiments was performed on stainless-steel coupons (ss2243, 2B) with 50 µl of a pure culture of *A. niger* or *B. cereus* spores or with *C. albicans* or *S. aureus*. All vegetative microbes were in a stationary phase but not starved. The number of microbes on the coupons varied between 10^5 and 10^8, depending on the organism. The *A. niger* spores were placed onto ice-cold stainless-steel coupons, allowed to dry at room temperature and then stored in the freezer to prevent the spores from germinating. The other organisms were allowed to dry at room temperature and then stored overnight at cold temperature. The coupons were prepared for about 18 h before the experiment.

These stainless-steel coupons were placed at different levels in a cheese storage room of approximately 30 m^3 in volume. After the treatment the coupons were removed aseptically. The coupons were placed as follows: a) on top of the highest shelf, b) on top of and beneath the midshelf and c) beneath the lowest shelf.

Before the disinfection treatments, i.e. ozone treatment and fogging, the ventilation was shut off and the experiment performed at room temperature. The ozone concentration was approximately 1 ppm and the relative humidity 43% in the first treatment, in the second treatment the parameters were 2 ppm and 53%, respectively. The fogging treatment was performed with a Disinfector 2000 (Clean Tech aps) device using both hydrogen peroxide and tenside based disinfectants. Three replicates of each microbe at each level were used. After the disinfection treatments the coupons were placed in a tube with neutralizer and shaken for 30 min and the number of microbes determined using culture technique. Tryptone glucose extract (TGE) agar was used for all microbes except *A. niger*, for which dichloranrose bengalchloramphenicol (DRBC) agar was used. The TGE agar plates were incubated for 2 d at 30 °C and the DRBC agar plates for 7 d at 20 °C. After the incubation, the number of colonies was enumerated.

5.8 Evaluation of Cleaning and Disinfection Efficacy of Various CIP Procedures using Spore-Soiled Surfaces

Stainless-steel surfaces were contaminated with *B. cereus* spores and milk. The test surfaces of stainless steel (ss2343, 2B; 45 x 45 mm^2) were soiled in 3 different ways:

- surfaces contaminated with *B. cereus* spores of the dairy strain 341. The spores were suspended in buffered saline solution (5 x 10^8 spores/ml) and sprayed with a paint-brush in 6 thin layers. After spraying the surfaces were dried overnight at room temperature in a sterile bench whose surfaces were gently rinsed 4 times in cold water just before the CIP cleaning.
- surfaces with *B. cereus* spores (NVH1) in milk. The test surfaces were mounted vertically in the spore-containing skim milk suspension, using a magnetic stirrer in the soiling vessel. Spores were allowed to adhere for 2 d at low temperature (4 °C). The surfaces with adhered spores were gently rinsed 4 times in cold water before the CIP cleaning.
- surfaces with milk-soil were soiled using 1 ml of 3% fat milk which was poured and spread, covering the entire surface, and then air-dried at 50 °C overnight. These surfaces were gently rinsed 4 times in cold water before the CIP cleaning.

After drying, the surfaces were cleaned and disinfected using 3 different CIP procedures in the vertical section of the pilot-plant CIP rig (120 l) at a flow of 1.5 m/s. A standard alkaline/acid procedure was compared with a formulated, enzyme-based agent, and to ozonated water (Ozotech, Norway). Ozone was produced and bubbled directly into the test-rig tank. Ozonated water was built up during 1–2 h. The conductivity was measured and the concentration of ozone was calculated from the oxidation-reduction potential (ORP) value. The concentrations of alkaline and acid substances (conductivity) were measured at-line during cleaning. The pH value was also determined by titration. It was not possible to determine the concentration of the enzyme-based agent either with conductivity or titration, and therefore an adequate amount of the agent was directly added to the buffer tank. The concentration of ozone was calculated using the ORP values. After cleaning and disinfection the number of surviving spores was enumerated and the amount of soil remaining estimated; all surfaces were rinsed with a neutralizing solution before the analyses were performed. The CIP procedures tested were:

- Standard rinse (prerinse with cold water for 5 min, 70 °C 0.9% NaOH for 10 min, 65 °C 1% nicric acid for 5 min and a final rinse with cold water for 5 min)
- Enzyme-based cleaning (prerinse with cold water for 5 min, treatment at 50 °C using 0.09% enzyme-based cleaner, rinse with cold water for 5 min, treatment with peroxygen-based 0.2% disinfectant and a final rinse with cold water for 5 min)
- Ozonated water (prerinse with cold water for 5 min, 0.5–1.0 ppm ozone rinse for 5–15 min and a final rinse with cold water for 5 min).

The number of surviving spores, i.e. the disinfection effect, was measured using swabbing of the surfaces and moulding with TTC agar. The amount of remaining milk-soil, i.e. the cleaning effect, was estimated in 5 different ways using a protein-measuring kit (SwabNCheck), ATP measurement, measurement of the contact angle of water, a UV microscopy method and staining the surfaces with fluorescent AO and microscopy.

5.9 TESTING OF CLEANING EFFICACY ON SURFACES

5.9.1 Process surfaces – Staining of *Bacillus* spores

To measure the number of *B. cereus* spores adhered to process surfaces after a cleaning process 3 methods were evaluated. The first, DEM, is based on staining the bacteria/spores with fluorochromes to visualize the organisms in a fluorescence microscope. In the DEM analysis the fluorochromes erythrosin B (ERB), acridine yellow (AY), auramine O (AU), pyridine derivative (C), AO and DAPI were used (Figs A–E; Appendix 15). However, the interaction of proteins and bacteria/spores causes problems in detection (Figs F–I; Appendix 15). The second method performed was indirect immunofluorescence, which is based on selective staining of *B. cereus* spores with fluorescence-stained antibodies. The method is specific to *B. cereus* spores and does not stain the milk proteins. Finally, a pilot test was performed with the QCM-DTM bioadhesion sensor. This is a new technique based on the measurements of the resonance frequency. The technique can be used for measurements of cell adhesion to different surface materials.

In the fluorochrome experiments the surfaces tested (stainless steel, glass, teflon, and silicone) were washed with ethanol and dried. The *B. cereus* spores (10^8 spores/ml) were prepared in three different mixtures: in saline and in milk-NaCl (1:10 and 1:50) solutions. The mixtures were spread onto surfaces by spraying twice. The surfaces were dried, whereafter they were stained with fluorochromes at different concentrations and the excess stains removed from the surfaces by rinsing with distilled water. The surfaces were then investigated using fluorescence microscopy. A total of 40 images (equivalent to 0.001 mm^2/surface) within each sample were calculated manually; the analysis was also performed automatically using an image analysis system (0.0081 mm^2/surface). The results were expressed in amount of spores/mm^2.

Glass surfaces (1 cm^2) were inoculated with spore suspension and dried for use in immunofluorescence studies. These surfaces were then placed in a humidity chamber. Phosphate buffered saline containing 1% bovine serum albumine was added and after a 15-min incubation at room temperature the excess buffer was removed. The primary antibody solution was added to the surfaces followed by incubation at 37 ºC for 60 min, washing with buffer and addition of the secondary antibody solution. These surfaces were incubated at 37 ºC for 30 min and washed with buffer before the surfaces were analysed with fluorescence microscopy. In all, 10 images (equivalent to a total of 0.625 mm^2) within the measurement frame were calculated manually, and the image analysis system calculated a total area of 0.529 mm^2. The experiments were performed with 2 different *B. cereus* strains, with and without addition of milk in the spore suspension (1:20 and 1:50, respectively). The results were expressed in amount of spores/ mm^2.

In the QCM-DTM the cell suspension was injected into the QCM chamber, which contained saline solution. The response was registered at the same moment as the cells were in contact with the sensor surface (gold). Both vegetative cells of *B. cereus* (3 x 10^8 cells/ml) and *B. cereus* spores (2 x 10^8 spores/ml) were examined. The measurements were performed twice without cleaning of the chamber between the 2 measurements.

5.9.2 Cleaning of a production line

Along the production line between pasteurization and packaging, a total of 55 sites were selected for enumeration of the total number of bacteria as well as *B. cereus*. The sites that could not be dismounted were swabbed with alginate swabs (LabDesign) and parts that could be dismounted were taken to SIK for moulding with TTC agar.

The swab assays were split into 2 parts. One part was directly analysed and the other part heat-treated (80 °C, 10 min) before analysing. The heat treatment was performed for the enumeration of *B. cereus* spores. The swab assays were inoculated on TGE and blood agar. All samples were incubated at room temperature for 14 d. Colonies with a *Bacillus* appearance were transferred for confirmation on *B. cereus* agar and Mossel agar.

Parts, e.g. sealings and gaskets, that were dismounted were moulded with Mossel agar containing polymyxin which is quite selective for *B. cereus*. The gaskets were split into 2 parts, one of which was moulded directly and the other heat-treated at 80 °C for 10 min before analysing. The colour indicator TTC was added to the agar just before moulding occurrred. These samples were incubated at room temperature for 14 d. Some colonies were transferred to TGE agar and then to *B. cereus* agar and Mossel agar for confirmation.

5.10 PREPARATION OF ENZYMES AND EFFICACY TESTING OF ENZYMATIC CLEANING

5.10.1 Preparation of proteinase

The following proteinase samples were prepared for comparison purposes in cleaning and disinfection in the dairy industry: cryotin from cod, trypsin from Antarctic krill, chymotrypsin from cod and alkalase from Novo. Profiles for pH optima for the enzymes were established to enable proper handling of the enzymes in cleaning and disinfection trials. Furthermore, activity assays, using low-molecular mass peptide substrates, were used to enable dosage of equivalent enzyme activity amounts in the cleaning and disinfecting trials. These activity assays were based on the amidolytic activity of the different enzymes: SucGPRpNA was used for cryotin from cod and trypsin from krill and

SucAAPFpNA was used for alkalase from Novo and chymotrypsin from cod. The measurements were performed at 25 °C using equilibrated buffer (Ásgeirsson *et al*., 1992; Bjarnason & Ásgeirsson, 1993; Bjarnason *et al*., 1997).

In the determinations the increase in absorption rate at 410 nm was recorded, and the activity corresponds to that section of the curve having the greatest slope. In some cases the initial rate can be low and the optimum is achieved in the second or third minute of the reaction, which is especially true for crude enzyme samples: one unit of enzyme activity is the amount of enzyme that hydrolyses 1 mmol of substrate per minute The comparative experiments were performed using equivalent activity concentrations of the enzymes in the comparative cleaning and disinfection experiments. Furthermore, a pH of approximately 8.0 was used in all experiments at refrigerated temperatures for 2 h and also extended duration of 24 h (Bjarnason & Ásgeirsson, 1993; Bjarnason *et al*., 1997).

5.10.2 Microtitration tray-based methods in efficacy testing

Various microtitration tray-based techniques were used to screen large numbers of samples for bacterial and chemical residues to enable choosing an optimal method for further work. The automated methods were:

- a fluorometric assay measuring residual bacterial metabolic activities using the fluorogenic redox substrate resazurin
- a colorimetric method analysing the effect of cleaning chemicals on dried milk and
- a turbidometric method measuring the lowest effective concentrations of the cleaning agents.

The fluorometric method for measuring residual bacterial metabolic activities was based on use of the fluorogenic redox substrate resazurin. The reaction was followed-up in a Fluoroskan II (Labsystems, Finland) for 60 h at 24 ºC. Resazurin is blue in the oxidized form and pink when reduced in the second stage of the reduction. Since the pink intermediate (resorufin) is fluorogenic, fluorometry can be used to monitor the resazurin reduction quantitatively. The concentration of residual protein on the microtitration trays after washing and rinsing was analysed using the Bio-Rad protein assay kit I. The residual

polysaccharides were measured using ruthenium red stain. The changes in turbidity using Bioscreen equipment (Labsystem) of the samples were measured to determine the bacterial growth, which can be seen as an increase in turbidity. This measurement was used to measure chemical residues in the samples after washing and rinsing. The fluorogenic method was chosen for use in further studies (Mattila-Sandholm *et al*., 1991).

6 CONCLUSIONS

6.1 CLEANING OF CLOSED SYSTEMS

The tests with the use of ozone for CIP cleaning and disinfection gave poor results in microbial reduction. The main reason for this is probably the failure of reaching an effective ozone concentration in the CIP system.

Although ozone is considered to be a very effective agent for disinfection there are still problems in achieving concentrations high enough in the CIP system. The CIP system in this study was too far away from the ozone generator and because of that the ozone decomposed in the air with reduced efficiency in disinfection. This shows clearly that all technical aspects of a method must be solved before the method is applied in the process.

6.2 COMPARISON OF TEST METHODS FOR DISINFECTANT EFFICIENCY

The bactericidal suspension tests cannot be used for comparison of commercial disinfectant brands or of sensitive microbes. Methods based on exposing microbes grown as biofilms and in biofilm-constructs to disinfectants provided information not only on the bactericidal effect per se, but also interactions between the disinfectants and the biofilm matrix.

6.3 TESTING OF AIR DISINFCTION IN INDUSTRIAL SCALE

In the tests performed there are differences between different types of disinfection methods but also between different chemical agents. Ozone was not to be as efficient on stressed microbes as it was on growing ones. In the case that the microbes were in a viable and growing phase they were quite easy to kill. Utilizing this step could make ozone more efficient. The trials also showed that it was important to have a high concentration and enough contact time.

When the disinfectant used contained hydrogen peroxide and peracetic acid the fogging was efficient while fogging with agents containing tensides was not efficient. There is a need to test new agents in a controlled way. These tests

should be based on surface tests. In performing surface test it is very important to know the behaviour of the microbes in different environments and situations.

6.4 Surface Disinfection in Industrial Scale

The disinfection efficiency of ozone on surfaces was not good. If the capacity of the equipment is sufficient and the surfaces are clean, fogging disinfection can be used to eliminate bacteria from surfaces. It is recommended that the disinfection efficacy in practical applications should be monitored frequently using several sampling sites.

6.5 Resistance Phenomena Due to Disinfection

Spores and some resistant vegetative bacteria can survive disinfection due to build-up of resistance against disinfectants. Some bacteria are intrinsically resistant whereas other adapt to the disinfectant used. It was also noticed that spores or bacteria forming biofilms may not be eliminated by ordinary cleaning and disinfection procedures.

6.6 Evaluation of the Cleaning of Cheese Moulds

The cheese moulds were washed with different agents and washing-methods and then analysed with different methods. None of the tested methods (protein-kit Check Pro, ATP, swabbing, washing out, UV-illumination, DEM and TTC agar) worked well for detection of soil. The TTC agar and washing out was most reliable for detection of microbial growth. These methods generate compatible and repeatable results. In the pilot-scale trials the contact agar method gave the most reliable results. There is a need for improvement of methods with which the level of organic residues can be detected so that the pure cleaning effect can be evaluated. In the full-scale trial the microbiological tests showed that the rinsing water was contaminated with a high number of bacteria. The methods can therefore also be used to evaluate parameters in the supply systems e.g. as the efficacy of the cleaning agents.

6.7 ENVIRONMENTAL ASSESSMENT

The main goal of cleaning and disinfection in the food industry is to remove dirt and fouling and to destroy any remaining microbes on the cleaned surfaces. Other aspects of cleaning are economy, environmental impact, safety and corrosion. Life cycle analysis (4.1) is a useful method for looking at the whole range of environmental impact of different CIP-methods, starting with raw materials and ending with effluent handling. The weakness of LCA in this context is the uncertainties in valuation and characterization of environmental impact from certain chemicals such as phosphonates and tensides.

A practical approach to evaluation of functionality of sanitation is described in 4.2. The steps consisting of assessment of written information, supplier audit, assessment of functional properties, practical evaluation and final judgement are suggested in such a process.

References

Ahmed, A. A.-H., Mustafa, M. K. & Marth, E. 1983a. Growth and enterotoxin production by *Staphyloccus aureus* in whey from the manufacture of Domiati cheese. J. Food. Prot., Vol. 46, pp. 235–237.

Ahmed, A. A.-H., Mustafa, M. K. & Marth, E. 1983b. Incidence of *Bacillus cereus* in milk and some milk products. J. Food. Prot., Vol. 46, pp. 126–128.

Anon. 1980. Kadova integral liner cheese moulds. Nordeuropeisk Mejeri-tidskrift, Vol. 46, pp. 36–39.

Anon. 1999. Bacics of ultrasonic cleaning technology. Finnsonic handbook. Lahti: Finnsonic Oy. Pp. 1–11.

Ásgeirsson, B. & Bjarnason, J. B. 1993. Properties of elastase from Atlantic cod, a cold adapted proteinase. Biochim. Biophys. Acta, Vol. 1164, pp. 91–100.

Ásgeirsson, B., Mäntylä, E. O. & Bjarnason, J. B. 1992. Purification and characterization of proteolytic digestive enzymes from the Pyloric Caeca of Atlantic Cod. In: Walther, B. T., Fyhn, H. J. & Jensen, A. M. (eds.). Development and aquaculture of marine larvae. Bergen: University of Bergen Press. Pp. 153–165.

Attaie, R., Whalen, R. J., Shahani, K. M. & Amer, M. A. 1987. Inhibition of *Staphylococcus aureus* during productionof Acidophilus yoghurt. J. Food Prot., Vol. 50, pp. 224–228.

Augustin, M. 2000. Rapid and sensitive fluorometric method for evaluation of the enzymatic cleaning agents against biofilm bacteria. Bachelor thesis. Helsinki: Pikku-Sitomo. 38 p. + 7 appendices.

Austin, J. W. & Bergeron, G. 1995. Development of bacterial biofilms in dairy processing lines. J. Dairy Res., Vol. 62, pp. 509–519.

Benkerroum, N., Oubel, H. & Ben Mimoun, L. 2002. Behavior of *Listeria monocytogenes* and *Staphylococcus aureus* in yoghurt fermented with a bacteriocin-producing thermophilic starter. J. Food Prot., Vol. 65, pp. 799–805.

Bertrand, F. 1987. The main steps in manufacture. In: Eck, A. (ed.). Cheesemaking: Science and Technology. Paris: Lavoisier Publishing. Pp. 413–421.

Bjarnason, J. B. & Ásgeirsson, B. 1993. Psychrophilic proteolytic enzymes from Atlantic Cod: their characteristics and applications. Gen. Eng. Biotechnol., Vol. 13, pp. 31–39.

Bjarnason, J. B., Kristjánsson, M. M., Ólafsdóttir, S., Benediktsson, B., Gudmundsdóttir, E. & Gudmundsdóttir, A. 1997. Characteristics and application of cryotin, a mixture of psychrotrophic marine proteinases. In: Hopsu-Havu, V. K. (ed.). Proteolysis in cell function. IOS Press. Pp. 104–111.

Blakistone, B., Chuyate, R., Kautter, D., Charbonneau, J. & Suit, K. 1999. Efficacy of Oxonia active against selected spore-formers. J. Food Prot., Vol. 63, pp. 262–267.

Block, S. 1991. Disinfection, sterilization and preservation, 4 ed. Philadelphia: Lea & Febiger.

Bloomfield, S. F. 1988. Cosmetics and pharmaceuticals: Biodeterioration and disinfectants. In: Houghton, D. R., Smith, R. N. & Eggins, H. O. W. (eds.). Biodeterioration 7. Essex: Elsevier Publishers Ltd. Pp. 135–145.

Bloomfield, S. F. 1992. Resistance of bacterial spores to chemical agents. In: Russel, A. D., Hugo, W. B. & Ayliffe, G. A. J. (eds.). Principles and practices of disinfection, preservation and sterilization. Oxford: Blackwell Scientific Publications. Pp. 230–245.

Bloomfield, S. F. & Arthur, M. 1994. Mechanisms in inactivation and resisitance of spores to chemical biocides. J. Appl. Bacteriol. Symp. Suppl., Vol. 76, pp. 91S–104S.

Bolton, K. J., Dodd, C. E. R., Mead, G. C. & Waites, W. M. 1988. Chlorine resistance of strains of Staphylococcus aureus isolated from poultry processing plants. Lett. Appl. Microbiol. Vol. 6, pp. 31–34.

Boulange-Peterman, L. 1996. Processes of bioadhesion on stainless steel surfaces and cleanability: a review with special reference to the food industry. Biofouling Vol. 10, pp. 275–300.

Brackett, R. E. 1992. Shelf stability and safety of fresh produce as influenced by sanitation and disinfection. J. Food Prot., Vol. 55, pp. 808–814.

Bradshaw, J. G., Peeler, J. T. & Twedt, R. M. 1991. Thermal resistance of Listeria spp. in milk. J. Food Prot., Vol. 54, pp. 12–14.

Bredholt, S., Maukonen, J., Kujanpää, K. Alanko, T., Olofson, U., Husmark U., Sjöberg, A.-M. & Wirtanen, G. 1999. Microbial methods for assessment of cleaning and disinfection of food-processing surfaces cleaned in a low-pressure system. Eur. Food Res. Technol., Vol. 209, pp. 145–152.

Brown, M. R. W. & Gilbert, P. 1993. Sensitivity of biofilms to antimicrobial agents. J. Appl. Bacteriol. Symp. Suppl., Vol. 74, pp. 87S–97S.

Bryers, J. D. & Weightman, A. 1995. The Centre for Biofilm Engineering: An international resource in managing complex biological systems. SIM News, Vol. 45, pp. 103–111.

Burfoot, D., Hall, K., Brown, K. & Xu, Y. 1999. Fogging for the disinfection of food processing factories and equipment. Food Sci. Technol., Vol. 10, pp. 203–210.

Bylund, G. 1995. Dairy processing handbook. Lund: LP Grafiska AB. Pp. 403–413.

Carpentier, B. & Cerf, O. 1993. Biofilms and their consequences, with particular reference to hygiene in the food industry. J. Appl. Bact., Vol. 75, pp. 499–511.

Carson, L. A., Favero, M. S., Bond, W. W. & Petersen, N. J. 1972. Factors affecting comparative resistance of naturally occurring and subcultured Pseudomonas aeruginosa to disinfectants. Appl. Microbiol., Vol. 23, pp. 863–869.

Chapman, H. R. & Sharpe, M. E. 1990. Microbiology of cheese. In: Robinson, R.K. (ed.). Dairy microbiology: The microbiology of milk products. Great Yarmouth: Elsevier Science Publishers Ltd. Pp. 203–291.

Characklis, W. G. 1981. Fouling biofilm development: a process analysis. Biotechnol. Bioeng., Vol. 23, pp. 1923–1960.

Chisti, Y. & Moo-Young, M. 1994. Cleaning-in-place systems for industrial bioreactors: design, validation and operation. J. Ind. Microbiol., Vol. 13, pp. 201–207.

Clark, R., Rice, E., Pierce, B., Johnson, C. & Fox, K. 1994. Effect of aggregation on Vibrio cholerae inactivation. J. Environ. Eng., Vol. 120, pp. 875–887.

Cloete, T. E., Smith, F. & Steyn, P. L. 1989. The use of planktonic bacterial populations in open and closed recirculating water cooling systems for the evaluation of biocides. Int. Biodeterior., Vol. 25, pp. 115–122.

Cnossen, H. J. & Wirtanen, G. 2002. HYFOMA European network for hygienic manufacturing of food & EHEDG Nordic. Poster presentation. Proceedings of the conference "The future for Nordic food innovation in a European context" 28–29 January 2002 in Stockholm. Oslo: Nordic Industrial Fund. 1 p.

Daufin, G., Ecalard, J. P. & Kerherve L. 1987. Cleaning and disifection. In: Eck, A. (ed.). Cheesemaking: Science and technology. Paris: Lavoisier Publishing. Pp. 444–449.

Davies, F. L. & Wilkinson, G. 1973. Bacillus cereus in milk and dairy products. In: Hobbs, B. C. & Christian, J. B. H. (eds.). The microbial safety of food. London: Academic Press. Pp. 57–67.

de Jong, P. 1997. Impact and control of fouling in milk processing. Trends Food Sci. Technol., Vol. 8, pp. 401–405.

Donnelly, C. W. 1994. Listeria monocytogenes. In: Hui, Y. H., Gorham, J. R., Murrel, K. D. & Cliver, D. O. (eds.). Foodborne disease handbook. Diseases caused by bacteria. New York: Marcel Dekker Inc. Pp. 215–252.

Donnelly, C. W. & Briggs, E. H. 1986. Psychrotrofic growth and thermal inactivation of Listeria monocytogenes as a function of milk composition. J. Food Prot., Vol. 49, pp. 994–998.

Driessen, F. M. & Loones, A. 1992. Developments in the fermentation process (liquid, stirred and set fermented milks). IDF Bull., Vol. 277, pp. 28–38.

Dunsmore, D. G., Twomey, A., Whittlestone, W. G. & Morgan, H. W. 1981. Design and performance of systems for cleaning product-contact surfaces of food equipment: a review. J. Food Prot., Vol. 44, pp. 220–240.

Dychdala, G. R. 1991. Chlorine and chlorine compounds. In: Disinfection, sterilisation and preservation, 4th Vol. Block, S. S. (ed.). Philadelphia: Lea & Febiger. Pp. 131–151.

European Hygienic Equipment Design Group (EHEDG). 1993a. Hygienic design of closed equipment for the processing of liquid food. Trends Food Sci. Technol., Vol. 4, pp. 375–379.

European Hygienic Equipment Design Group (EHEDG). 1993b. Hygienic equipment design criteria. Trends Food Sci. Technol., Vol. 4, pp. 225–229.

European Hygienic Equipment Design Group (EHEDG). 1994. Hygienic design of valves for food processing. Trends Food Sci. Technol., Vol. 5, pp. 169–171.

Exner, M., Tuschewitzki, G.-J. & Scharnagel, J. 1987. Influence of biofilms by chemical disinfectants and mechanical cleaning. Zbl. Bakteriol, Mikrobiol. Hygiene, Serie B, Vol. 183, pp. 549–563.

Farber, J. M. & Peterkin, P. I. 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiol. Rev., Vol. 55, pp. 476–511.

Farber, J. M., Daley, E., Coates, F., Emmons, D. B. & McKellar, R. 1992. Factors influencing survival of *Listeria monocytogenes* in milk in a high-temperature short-time pasteurizer. J. Food Prot., Vol. 55, pp. 946–951.

Flemming, H.-C. 1991. Biofouling in water treatment. In: Biofouling and biocorrosion in industrial water systems. Flemming, H.-C. & Geesey, G. G. (eds.). Berlin–Heidelberg: Springer–Verlag. Pp. 47–80.

Flemming, H.-C., Schaule, G. & Mcdonogh, R. 1992. Biofouling on membranes – a short review. In: Biofilms – Science and Technology. Melo, L. F., Bott, T. R., Fletcher, M. & Capdeville, B. (eds.). Dordrecht: Kluwer Academic Publishers. Pp. 487–497.

Flint, S. H., Bremer, P. J. & Brooks, J. D. 1997. Biofilms in dairy manufacturing plant – description, current concerns and methods of control. Biofouling, Vol. 11, pp. 81–97.

Fox, P. F. 1987. Cheese: An overview. In: Fox, P. F. (ed.). Cheese: Chemistry, physics and microbiology. New York: Elsevier Applied Science. Vol. 1. Pp. 1–33.

Frandsen, T. 1992. Milk for cheese production. Scand. Dairy Inf., Vol. 1, pp. 12–13.

Gilbert, P., Jones, M. V., Allison, D. G., Heys, S., Maira, T. & Wood, P. 1998. The use of poloxamer hydrogels for the assessment of biofilm susceptibility towards biocide treatments. J. Appl. Microbiol., Vol. 85, pp. 985–991.

Grönholm, L., Wirtanen, G., Ahlgren, K., Nordström, K. & Sjöberg, A.-M. 1999. Screening of antimicrobial activities of disinfectants and cleaning agents against foodborne spoilage microbes. Z. Lebensm. Unters. Forsch. A, Vol. 208, pp. 289–298.

Hancock, D. D., Besser, T. E., Kinsel, M. L., Tarr, P. I., Rice, D. H. & Paras, M. G. 1994. The prevalence of *Eschericia coli* in dairy and beef cattle in Washington State. Epidemiol. Infect., Vol. 113, pp. 199–207.

Hansen, R. 1986. Surface cleaning of dairy equipment. North Europ. Dairy J., 10, p. 285.

Hasting, T. 1995. Good enough to eat. The Chemical Engineer, 9 Nov. 1995, pp. 19–20.

Heeschen, W. & Hahn, G. 1996. Escherichia coli: alter Freund – neuer Feind? Deutsche Milchwirtschaft, Vol. 10, pp. 448–454.

Heino, A. 2000. Juustomuottien puhtauden tutkiminen pilot- ja prosessimitta-kaavassa (Cleanliness of cheese moulds in pilot and process scale). EKT-series 1182. Helsinki: Pikku-Sitomo. 117 p.

Heinzel, M. 1988. The phenomena of resistance to disinfectants and preservatives. In: Industrial Biocides. Chichester: John Wiley & Sons. Pp. 52–67.

Heinzel, M. & Bellinger, H. 1982. Microbiological investigations with decentral dosing apparatures for disinfectants. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. I. Orig., Vol. 176, pp. 47–54.

Helander, I. M., Alakomi, H.-L., Latva-Kala, K. & Koski, P. 1997. Polyethyleneimine is an effective permeabilizer of Gram-negativebacteria. Microbiol., Vol. 143, pp. 3193–3199.

Helander, I. M. & Mattila-Sandholm, T. 2000. Fluorometric assessment of Gram-negative bacterial permeabilization. J. Appl. Microbiol., Vol. 88, pp. 213–219.

Holah, J. T. 1992. Industrial monitoring: hygiene in food processing. In: Biofilms – Science and Technology. Melo, L. F., Bott, T. R., Fletcher, M. & Capdeville, B. (eds.). Dordrecht: Kluwer Academic Publishers. Pp. 645–659.

Holah, J. & Gibson, H. 1999. Biofouling: cleanability problems in food processing? In: 30th R^3-Nordic Contamination Control Symposium. Wirtanen, G., Salo, S. & Mikkola, A. (eds.). Espoo: VTT Symposium 193. Pp. 177–186.

Holah, J., Lavaud, A., Peters, W. & Dye, K. 1998. Future techniques in disinfectant testing. Int. Biodeterior. Biodegrad., Vol. 41, pp. 273–279.

Holah, J. & Timperley, A. 1999. Hygienic design of food processing facilities and equipment. In: 30th R^3-Nordic Contamination Control Symposium. Wirtanen, G., Salo, S. & Mikkola, A. (eds.). Espoo: VTT Symposium 193. Pp. 11–39.

Hood, S. K. & Zottola, E.A. 1995. Biofilms in food processing. Food Control, Vol. 6, pp. 9–18.

Husmark, U. 1993. Adhesion mechanisms of bacterial spores to solid surfaces. Göteborg: Chalmers Biblioteks ReproService. 35 p. + 5 appendices. ISBN 91-7032-778-5.

Härkönen, P., Salo, S., Mattila-Sandholm, T., Wirtanen, G., Allison, D. G. & Gilbert, P. 1999. Development of a simple in vitro test system for the disinfection of bacterial biofilms. Water Sci. Technol., Vol. 39, pp. 219–225.

Johnson, E. A., Nelson, J. H. & Johnson, M. 1990. Microbial safety of cheese made from heat-treated milk. Part II. Microbiology. J. Food Prot., Vol. 53, pp. 519–540.

Keene, W. E., Hedberg, K., Herriott, D. E., Hancock, D. D., McCay, R. W., Barrett, T. J. & Flemming, D. W. 1997. A prolonged outbreak of *Escherichia coli* O157:H7 infections caused by commercially distributed raw milk. J. Infect. Dis., Vol. 176, pp. 815–818.

Kellett, B. S. 1979. Bacterial growth in amphylobactic disinfectant solution. Laboratory animals, Vol. 13, pp. 135–138.

Kessler, H. G. 1981. Food engineering and dairy technology. Freising: Verlag A. Kessler.

Kinniment, S. & Wimpenny, J. W. T. 1990. Biofilms and biocides. Int. Biodeterior., Vol. 26, pp. 181–194.

Kivelä, T. 1996. Easier cheese mould cleaning by ultrasonics. Scand. Dairy Inf., Vol., 1, pp. 34–35.

Koivisto, M. 1999. Oral information. Lapinlahti: Valio Ltd.

Langsrud, S., Baardsen, B. & Sundheim, G. 2000. Potentiation of the lethal effect of peroxygen on Bacillus cereus spores by alkali and enzyme wash. Int. J. Food Microbiol., Vol. 56, pp. 81–86.

Langsrud, S. & Møretrø, T. 2001. Characterisation of bacteria surviving in disinfecting footbaths. SFAM summer Conference 2001, 9–12 July, Swansea (UK). Poster abstract. 1 p.

Larson, E. L. & Morton, H. E. 1991. Alcohols. In: Disinfection, sterilisation and preservation, 4th Vol. Block, S. S. (ed.). Philadelphia: Lea & Febiger. Pp. 204–214.

LeChevallier, M. W., Cawton, C. D. & Lee, R. G. 1988. Inactivation of biofilm bacteria. Appl. Environ. Microbiol., Vol. 54, pp. 2492–2499.

LeChevallier, M. W., Lowry, C. D. & Lee, R. G. 1990. Disinfecting biofilms in a model distribution system. Am. Water Works Assoc. J., Vol. 827, pp. 87–99.

Lovett, J., Wesley, I. V., Vandermaaten, M. J., Bradshaw, J. G., Francis, D. W., Crawford, R. G., Donnelly, C. W. & Messer, J. W. 1990. High-temperature short-time pasteurization inactivates *Listeria monocytogenes*. J. Food Prot., Vol. 53, pp. 734–738.

Lowbury, E. 1951. Contamination of cetrimide and other fluids with *Pseudomonas pyocyanea*. Br. J. Ind. Med., Vol. 8, pp. 22–25.

Mackey, B. M. & Bratchell, N. 1989. The heat resistance of *Listeria monocytogenes*. A review. Lett. Appl. Microbiol., Vol. 6, pp. 89–94.

Marrie, T. J. & Costerton, J. W. 1981. Prolonged survival of *Serratia marcescens* in chlorhexidine. Appl. Environ. Microbiol., Vol. 42, pp. 1093–1102.

Matalon, M. E. & Sandine, W. E. 1986. *Lactobacillus bulgaricus, Streptococcus thermophilus* and yoghurt: a review. Cult. Dairy Prod. J., Vol. 21, pp. 4:6–12.

Mattila, T., Manninen, M. & Kyläsiurola, A.-L. 1990. Effect of cleaning-in-place disinfectants on wild bacterial strains isolated from a milking line. J. Dairy Res., Vol. 57, pp. 33–39.

Mattila-Sandholm, T., Ali-Vehmas, T., Wirtanen, G., Rönner, U. & Sandholm, M. 1991 Automated fluorometry in quality control of pasteurized and ultra-high temperature-treated starch soup. Int. J. Food Sci. Technol., Vol. 26, pp. 325–336.

Mattila-Sandholm, T. & Wirtanen, G. 1992. Biofilm formation in the industry, a review. Food Rev. Int., Vol. 8, pp. 573–603.

McDonnell, G. & Russell, D. 1999. Antiseptics and disinfectants: activity, action and resistance. Clin. Microbiol. Rev., Vol. 12, pp. 147–179.

McKane, L. & Kandel, J. 1996. Microbiology: Essentials and applications. 2nd ed. New York: McGraw–Hill Inc. Pp. 76–83.

Mead, G. C. & Adams, B. W. 1986. Chlorine resistance of Staphylococcus aureus isolated from turkeys and turkey products. Lett. Appl. Microbiol., Vol. 3, pp. 131–133.

Mechie, S. C., Chapman, P. A. & Siddons, C. A. 1997. A fifteen month study of *Escherichia coli* O157:H7 in a dairy herd. Epidemiol. Infect., Vol. 118, pp. 17–25.

Mettler, E. & Carpentier, B. 1998. Varations over time of microbial load and physicochemical properties of floor materials after cleaning in food industry premises. J. Food Prot., Vol. 61, pp. 57–65.

Mikkola, J. 1999. Uusia seulontamenetelmiä puhdistusaineiden tehon mittaamiseksi meijeripesuja varten. – Fluorometrinen, turbidometrinen ja kolorimetrinen menetelmä (New screening methods for the analysis of the efficiency of enzymatic cleaning agents used in dairies). Bachelor thesis, Helsinki. 58 p. + 9 appendices.

Miller, A. J., Whiting, R. C. & Smith, J. L. 1997. Use of risk assessment to reduce listeriosis incidence. Food Technol., Vol. 51, pp. 100–103.

Morton, L., Greenway, D., Gaylarde, C. & Surman, S. 1998. Considerations of some implications of the resistance to biofilms to biocides. Int. Biodeterior. Biodegrad., Vol. 41, pp. 247–259.

Mosteller, T. M. & Bishop, J. R. 1993. Sanitizer efficacy against attached bacteria in a milk biofilm. J. Food Prot., Vol. 56, pp. 34–41.

Mucchetti, G. 1995. Biological fouling and biofilm formation on membranes. Int. Dairy Fed. Special Issue No. 9504, pp. 118–124.

Murinda, S. E., Nguyen, L. T., Ivey, S. J., Gillespie, B. E., Almeida, R. A., Draughon, F. A. & Oliver, S. P. 2002. Prevalence and molecular characterization of *Escherichia coli* O157:H7 in bulk tank milk and fecal samples from cull cows: a 12-month survey of dairy farms in east Tennessee. J. Food Prot., Vol. 65, pp. 752–759.

Nichols, W. W. 1991. Biofilms, antibiotics and penetration. Rev. Med. Microbiol., Vol. 2, pp. 177–181.

Nicoletti, G., Boghossian, F., Gurevitch, F., Borland, R. & Morgenroth, P. 1993. The antimicrobial activity in vitro of chlorhexidine, a mixture of iso-thiazolinones ('Kathon' CG) and cetyl trimethyl ammonium bromide (CTAB). J. Hosp. Infect., Vol. 23, pp. 87–111.

Pirbazari, M., Voice, T. C. & Weber, W. J. Jr. 1990. Evaluation of biofilm development on various natural and synthetic media. Hazard, Waste Hazard. Mater., Vol. 7, pp. 239–250.

Price, D. & Ahearn, D. G. 1988. Incidence and persistence of Pseudomonas aeruginosa in whirlpools. J. Clin. Microbiol., Vol. 26, pp. 1650–1654.

Russel, A. D. 1990. Bacterial spores and chemical sporicidal agents. Clin. Microbiol. Rev., Vol. 3, pp. 99–119.

Russell, A. & Chopra, I. 1996. Understanding antibacterial action and resistance, 2 edn. London: Ellis Horwood. Pp. 150–171.

Savello, P. A. & Dargan, R. A. 1995. Improved yoghurt physical properties using ultrafiltration and very-high temperature heating. Milchwissenschaft, Vol. 50, pp. 86–90.

Sinha, R. P., Modler, H. W. & Emmons, D. B. 1989. Changes in acidity and starter bacteria in commercial yoghurts during storage. Cult. Dairy Prod. J., Vol. 24, pp. 2:12–16.

Storgårds, E., Simola, H., Sjöberg, A.-M. & Wirtanen, G. 1999. Hygiene of gasket materials used in food processing equipment. Part 2: Aged materials. Trans. Inst. Chem. Eng. Part C, Vol. 77, pp. 146–155.

Sundheim, G. & Langsrud, S. 1995. Natural and acquired resistance of bacteria associated with food processing environments to disinfectant containing an extract from grapefruit seeds. Int. Biodeterior. Biodegrad., Vol. 36, pp. 441–448.

Tamine, A. Y. 1993. Modern cheesemaking: hard cheeses. In: Robinson, R. K. (ed.). Modern dairy technology – Advances in milk products. Cambridge: Blackie Academic Professional. Pp. 49–221.

Te Giffel, M. C., Beumer, R. R., van Dam, W. F., Slaghuis, B. A. & Rombouts, F. M. 1995. Sporicidal effect of disinfectants on Bacillus cereus isolated from milk processing environment. Int. Biodeter. Biodegrad., Vol. 36, pp. 421–430.

Troller, J. A. 1993. Sanitation in food processing. San Diego: Academic Press, Inc. Pp. 30–70 & pp. 263–286.

Tuncan, E. U. 1993. Effect of cold temperature on germicidal efficacy of quaternary ammonium compound, iodophor, and chlorine on Listeria. J. Food Prot., Vol. 56, pp. 1029–1033.

Vaara, M. 1992. Agents that increase the permeability of the outer membrane. Microbiol. Rev., Vol. 56, pp. 395–411.

Visser, H. 1997. Fouling and cleaning of heat equipment. IDF Bull. Doc., Vol. 328, p. 4.

Visser, H. & Jeurnink, Th. J. M. 1997. General aspects of fouling and cleaning. IDF Bull. Doc., Vol. 328, pp. 5–6.

Waites, W. M. & Bayliss, C. E., 1980. The preparation of bacterial spores for evaluation of sporicidal activity of chemicals. In: Gould, G. W. & Corry, J. E. L. (eds.). Microbial growth and survival in extremes of environment. London: Academic Press. Pp. 159–172.

Wirtanen, G. 1995. Biofilm formation and its elimination from food processing equipment. Espoo: VTT Publications 251. 106 p. + appendices 48 p. ISBN 951-38-4789-6.

Wirtanen, G. & Mattila-Sandholm, T. 2002. Biofilms in the food industy. In: Encyclopedia of Environmental Microbiology, Bitton, G. (ed.). New York: John Wiley & Sons, Inc. In press.

Wirtanen, G., Salo, S., Allison, D., Mattila-Sandholm, T. & Gilbert, P. 1998. Performance-evaluation of disinfectant formulations using poloxamer-hydrogel biofilm-constructs. J. Appl. Microbiol., Vol. 85, pp. 965–971.

Wirtanen, G., Salo, S., Helander, I. M. & Mattila-Sandholm, T. 2001. Microbiological methods for testing disinfectant efficiency on Pseudomonas biofilm. Colloids and Surfaces B: Biointerfaces, Vol. 20, pp. 37–50.

Wirtanen, G., Salo, S., Maukonen, J., Bredholt, S. & Mattila-Sandholm, T. 1997. NordFood Sanitation in dairies. Espoo: VTT Publications 309. 47 p. + appendices 22 p. ISBN 951-38-5055-2.

Wirtanen, G., Saarela, M. & Mattila-Sandholm, T. 2000. Biofilms – Impact on hygiene in food industries. In: Biofilms II: Process Analysis and Applications, Bryers, J. (ed.). New York: John Wiley–Liss Inc. Pp. 327–372. ISBN 0-471-29656-2.

Wong, H.-C., Chang, M.-H. & Fan, J.-Y. 1988. Incidence and characterization of Bacillus cereus isolates contaminating dairy products. Appl. Environ. Microbiol., Vol. 54, pp. 699–702.

Wood, P., Jones, M. Bhakoo, M. & Gilbert, P. 1996. A novel strategy for control of microbial biofilms through generation of biocide at the biofilm-surface interface. Appl. Environ. Microbiol., Vol. 62, pp. 2598–2602.

Zottola, E. A. & Sasahara, K. C. 1994. Microbial biofilms in the food processing industry – should they be a concern? Int. J. Food Microbiol., Vol. 23, pp. 125–148.

Zottola, E. A. & Smith, L. B. 1991. Pathogens in cheese. Food Microbiol., Vol. 8, pp. 171–182.

Zuniga-Estrada, A., Lopez-Merino, A. & Mota de la Garza, L. 1995. Behavior of *Listeria monocytogenes* in milk fermented with a yoghurt starter culture. Rev. Latinoam. Microbiol., Vol. 37, pp. 257–265.

Summary of activities in the project.

TASK	FINLAND	NORWAY	SWEDEN	ICELAND
1 Ozone, fogging, foot baths		• Standardized method for ozonation & fogging (evaluation of the disinfection effect) -ozone: Arla (SIK) -fogging: TINE (Matforsk) Meeting 5.10.1998 with SIK, Arla, Tine and Matforsk • Foot bath work Matforsk and TINE • Evaluation of the test method Matforsk & TINE • Tolerance of microbes after ozonation and in foot baths • Ozone in water • Listeria-strains in foot bath		
2 Cheese moulds -methods -evaluation	• Student work based on these methods at VTT in cooperation with Valio during August 1999 - March 2000		• Process scale trials -Arla & SIK • Meeting at SIK and Bergen	
3. Enzymes -lab scale -pilot scale -cleaning agents: Novadan, Novo, DL, Henkel	• Student works at University of Helsinki (HU): cleaning in cold areas	• Paradigme study in lab scale at Matforsk • Commercial agents to HU • Paradigme study with alternative method at Tine • Paradigme study: following up the practical effect in dairy		Several types of enzymes • cryotin (cod) • chymotrysin (cod) • trysin (krill) • trysin (herring) (mixture) • capelin (for lab scale tests at HU)
4. CIP cleaning, pasteurizer -ozone -methods (DEM)	• graduate work: yoghurt line • pasteurizer • soiling equipment designed by Urban	• ozone (TINE) • Testrig tests in SIK	CIP-cotrials together with Matforsk	
5 Ice water (cooling after pasteurizing) -microbes -corrosion	• samples from Haapajoki • initial trials at VTT using ozone			
6 Methods:	• visit to Center of Biofilm Engineering (microscopy) • visit to Manchester University (hydrogel) Studentworks: hydrogel	to study tolerance starving natural resistance	studentwork: DEM & fluorochromes	
7 Procedures for evaluating		LCA	How to evaluate function environmentaly effect by Arla	

APPENDIX 1

Poster presentation Highlights from the NordFood2 Project P96049 Evaluation of cleaning agents and disinfectants for use in dairies: Methods and mechanisms (1997–2000).

PUBLICATIONS, ORAL AND POSTER PRESENTATIONS AS WELL AS THESIS

ORIGINAL ARTICLES, BOOK CHAPTERS, ORAL AND POSTER PRESENTATIONS

1997

- Gilbert, P., Wirtanen, G. & Allison, D. (1997). Standard laboratory test methods and their relevance in the evaluation of disinfectants. Autumn Meeting of the Society for Applied Microbiology (October 22, 1997). SFAM, London. 2 p.
- Wirtanen, G., Salo, S., Maukonen, J., Bredholt, S. & Mattila-Sandholm, T. (1997). NordFood Sanitation in dairies. Espoo: VTT Publications 309. 47 p. + appendices 22 p. ISBN 951-38-5055-2.

1998

- Wirtanen, G., Salo, S., Allison, D., Mattila-Sandholm, T. & Gilbert, P. (1998). Performance-evaluation of disinfectant formulations using poloxamer-hydrogel biofilm-constructs. Journal of Applied Microbiology, 85, pp. 965–971.
- Mikkola, J., Ahlgren, J. & Ali-Vehmas, T. (1998). A fluorometric screening method for analysing bacterial activities in biofilms – Efficacy of enzymatic cleaning procedures. Tiedevinkki, Faculty of Veterinary Medicine, University of Helsinki (Poster presented Dec. 12, 1998).

1999

- Bredholt, S., Maukonen, J., Kujanpää, K., Alanko, T., Olofson, U., Husmark, U., Sjöberg, A.-M. & Wirtanen, G. (1999). Microbial methods for assessment of cleaning and disinfection of food-processing surfaces cleaned in a low-pressure system. European Food Research and Technology, 209, pp. 145–152.
- Härkönen, P., Salo, S., Mattila-Sandholm, T., Wirtanen, G., Allison, D.G. & Gilbert, P. (1999). Development of a simple *in vitro* test system for the disinfection of bacterial biofilms. Water Science and Technology, 39, pp. 219–225.
- Høgaas Eide, M. & Homleid, J. P. (1999). Livsløpsanalyse (LCA) av CIP-metoder. Meieriposten nr. 2/99, pp 32–35.

- Sundheim, G. & Eide, O. K. (1999). Bakteriologiske undersøkelser av fotbad. Meieriposten, 7, pp. 190–192.
- Sundheim, G. & Homleid, J. P. (1999). Forhold som påvirker effekten av tåkedesinfeksjon. Meieriposten, 9, pp. 248–255.
- Wirtanen, G., Salo, S. & Mattila-Sandholm, T. (1999). Microbial methods in evaluation of cleaning procedures. In Wirtanen, G., Salo, S. & Mikkola, A. (eds.). 30th R^3-Nordic Contamination Control Symposium. VTT Symposium 193. Libella Painopalvelu Oy, Espoo. Pp. 207–218.

2000

- Langsrud, S., Baardsen, B. & Sundheim, G. (2000). Potentiation of the lethal effect of peroxygen on *Bacillus cereus* spores by alkali and enzyme wash. International Journal of Food Microbiology, 56, pp. 81–86.
- Maukonen, J., Mattila-Sandholm, T. & Wirtanen, G. (2000). Metabolic indicators for assessing bacterial viability in hygiene sampling using cells in suspensions and swabbed biofilm. Lebensmittel-Wissenschaft und -Technologie, 33, pp. 225–234.
- Willcock, L., Allison, D. G., Holah, J., Wirtanen, G. & Gilbert, P. (2000). Influence of fluid dynamic forces upon the steady-state population dynamics in microbial biofilm communities. Journal of Industrial Microbiology, 25, pp. 235–241.
- Wirtanen, G., Salo, S., Aalto, M. & Gilbert, P. (2000). Disinfectant testing using microbes grown in biofilm-constructs. In: Proceedings of 15th ICCCS International Symposium and 31st R^3-Nordic Symposium on Contamination Control. Pp. 319–324.
- Wirtanen, G., Heino, A. & Salo, S. (2000). Ultrasound cleaning in cheese mold hygiene. Proceedings of the 87th Annual Meeting of International Association for Food Protection. Atlanta, Georgia. Poster P9. Pp. 40.
- Wirtanen, G., Kontulainen, S. & Salo, S. (2000). Effects of cleaners of biofouled stainless-steel surfaces in yoghurt manufacturing equipment. Proceedings of the 87th Annual Meeting of International Association for Food Protection. Atlanta, Georgia. Poster P6. Pp. 39.
- Wirtanen, G., Saarela, M. & Mattila-Sandholm, T. (2000). Biofilms – Impact on hygiene in food industries. In Biofilms II: Process Analysis and Applications, Bryers, J. (ed.). New York: John Wiley-Liss Inc. Pp. 327–372. ISBN 0-471-29656-2.

- Wirtanen, G., Salo, S., Aalto, M. & Gilbert, P. (2000). Disinfectant testing using microbes grown in poloxamer-hydrogel biofilm-constructs. Proceedings of the ASM Conference on Biofilm 2000 (B2K). Big Sky, Montana. Poster 113. Pp. 55.
- Wirtanen, G., Storgårds, E., Saarela, M., Salo, S. & Mattila-Sandholm, T. (2000). Detection of Biofilms in the Food and Beverage Industry. In Industrial Biofouling, Walker, J., Surman, S. & Jass, J. (eds.). John Wiley & Sons, Ltd., Chichester. Pp. 175–203. ISBN-0-471-98866-9.

2001

- Bore, E. & Langsrud, S. (2001). Identification of microorganisms isolated from dairy industy after cleaning and disinfection. SFAM Summer Conference, 9–12 July, Swansea (UK). Poster abstract. 1 p.
- Langsrud, S. & Møretrø, T. (2001). Characterisation of bacteria surviving in disinfecting footbaths. SFAM Summer Conference 2001, 9–12 July, Swansea (UK). Poster abstract. 1 p.
- Wirtanen, G., Salo, S., Helander, I. M. & Mattila-Sandholm, T. (2001). Microbiological methods for testing disinfectant efficiency on *Pseudomonas* biofilm. Colloids and Surfaces B: Biointerfaces, 20, pp. 37–50.
- Wirtanen, G., Aalto, M., Härkönen, P., Gilbert, P. & Mattila-Sandholm, T. (2001). Efficacy testing of commercial disinfectants against foodborne pathogenic and spoilage microbes in biofilm-constructs. European Food Research and Technology, 213, 409–414.

2002

- Wirtanen, G. (2002). Highlights from the NordFood2 Project P96049 "Evaluation of cleaning agents and disinfectants for use in dairies: Methods and mechanisms" (1997–2000). Symposium on "The future for Nordic food innovation in a European context" in Stockholm 28–29 January 2002. Oslo: Nordic Industrial Fund. Poster abstract. 2 p.
- Wirtanen, G., Salo, S., Heino, A., Hattula, T. & Mattila-Sandholm, T. (2002). Comparison of ultrasound based cleaning programs for cheesery utensils. In: Fouling, Cleaning and Disinfection in Food Processing. Wilson, D. I., Fryer, P. J. & Hastings, A. P. M. (eds.). Cambridge: City Services Design and Print. Pp. 165–171. ISBN 0 9542483 0 9.

- Wirtanen, G., Salo, S. & Storgårds, E. 2002. Microbial assessment methods.used.in cleaning efficacy evaluation. In: Matuszek, T. (ed.). Food, packaging, equipment and building surfaces in their contribution to food products contamination and process safety. Gdansk: Gdansk University of Technology Publishing Office. Pp. 49–57. ISBN 83-88579-40-1.
- Sundheim, G. & Langsrud, S. (2002). Characterization of *Cedecea* like strains surviving in disinfecting footbath. Manuscript in preparation.

THESIS OF GRADUATE STUDENTS

1997

- Kallio, J. (1997). Gram-negatiivisten bakteerien solunläpäisevyyttä edistävien aineiden vaikutukset pesu- ja desinfiointiaineiden tehokkuuteen (The effect of permeability-increasing agents and disinfectants on Gram-negative bacteria). Bachelor thesis, Helsinki: Pikku-Sitomo. 75 p. + 5 appendices.

1999

- Härkönen, P. (1999). *In vitro* -testin kehittäminen desinfiointiaineiden tehon testaamiseen biofilmibakteereilla (Development of an *in vitro* test system for disinfection of biofilm bacteria). Bachelor thesis, Helsinki: Pikku-Sitomo. 60 p. + 1 appendix.
- Mikkola, J. (1999). Uusia seulontamenetelmiä puhdistusaineiden tehon mittaamiseksi meijeripesuja varten. – Fluorometrinen, turbidometrinen ja kolorimetrinen menetelmä (New screening methods for the analysis of the efficiency of enzymatic cleaning agents used in dairies). Bachelor thesis, Helsinki: EVTEK. 58 p. + 9 appendices.
- Nilsson, A. (1999). Detektion av Bacillus cereus sporer på yta med hjälp av DEM, immunofluorescens och QCM-D™ (Detection of *Bacillus cereus* spores on surfaces using DEM, immunofluorescence and QCM-D™). Master thesis 1999:L4, Kalmar. 33 p. + 5 appendices.

2000

- Kontulainen, S. (2000). Termofiilisten haittabakteerien vaikutus jogurtin kypsymisen hidastumiseen ja valmistuslaitteistojen pintojen peseytyvvyyteen (Effect of harmful thermophilic bacteria in yoghurt fermentation and cleaning of surfaces in manufacturing equipment). Pro Gradu thesis (University of Helsinki), EKT-series 1175, Pikku-Sitomo, Helsinki. 89 p.
- Heino, A. (2000). Juustomuottien puhtauden tutkiminen pilot- ja prosessi-mittakaavassa (Cleanliness of cheese moulds in pilot and process scale). Pro Gradu thesis (University of Helsinki), EKT-series 1182, Pikku-Sitomo, Helsinki. 117 p.
- Aalto, M. (2000). Desinfiointiaineiden teho biofilmimikrobeihin (Disinfectant efficacy against microbes in biofilm-constructs). Bachelor thesis, Helsinki, Pikku-Sitomo. 66 p.
- Augustin, M. (2000). Rapid and sensitive fluorometric method for evaluation of the enzymatic cleaning agents against biofilm bacteria. Bachelor thesis, Pikku-Sitomo, Helsinki. 38 p. + 7 appendices.

PROJEKTSAMMANDRAG (SVENSKA)

Forskningsarbetet i projektet P96049 inom ramprogrammet NORDFOOD2 utfördes vid de nordiska forskningsinstituten VTT Bioteknik, MATFORSK och SIK samt vid Helsingfors och Reykjavik universitet fr.o.m. april 1997 t.o.m. januari 2000. De deltagande företagen var mejerierna Valio från Finland, Arla från Sverige och TINE från Norge samt teknokemiföretaget Suomen Unilever Oy DiversyLever från Finland. Dr. Gun Wirtanen, VTT Bioteknik, koordinerade projektet. Maija Uusisuo och Oddur Gunnarsson skötte om projektet från Nordisk industri fonds sida. Experimenten, vilka utfördes i projektet, fokuserde på mätningsmetoder inom rengöring av öppna och slutna system, t.ex. dim-desinfektion, ozonering, fotbadshygien, rengöring av ostformar och yoghurtlinjer samt utveckling av metoder för testning av desinfektionsmedels effekt, mikrobiologiska resistensfenomen, livscykelanalyser (LCA) och rengörings-procedurens funktionsduglighet. Nya hygienlösningar baserade på projektresultaten har införts i mejerierna. Projektresultaten kan summeras enligt följande:

- Det huvudsakliga målet i det svenska projektet var att utveckla och utvärdera praktiska metoder för mätning av hur effektiv rengörings- och desinfektion-processen är. Kravet var att metoderna skulle kunna användas på ytor i olika typers mejeriapparater. Analysmetoder baserade på utsköljning och ingjutning med trifenyltetrazoliumklorid (TTC) agar fungerade bra för testning av rengöringen av plast ostformar. En 5-stegs metod för evaluering av rengörings- och desinficeringsmedel har utvecklats på Arla Foods.

- *Bacillus cereus* är en viktig mikrob vid desinfektion av utrustning som används i produktion av konsumtionsmjölk i Norden. Totala antalet *Bacillus* sporer på hårda ytor är en användbar indikator vid mätning av rengöringseffekten. Förbättrade detektionsmetoder för mätning av sporer fästade vid ytor kan grunda sig på fluorescerande färgämnen, indirekt immunofluorescens och kvartskristallmikrobalansteknik.

- Miljövänliga rengöringsprocedurer, där ozonerat vatten eller enzymbaserade rengöringsmedel används, utvärderades i ett CIP-system. I dessa metoder kan man spara energikostnader i tillägg till mindre förorening av miljön. Lovande

resultat med en sänkning av spormängden på 1–3 log-enheter (90,0–99,9%) uppnåddes då ozonkoncentration var 0,1–0,3 ppm.

- Dimdesinfektion och ozonering är potentiella metoder för desinficering av luft och ytor. Det uppkom emellertid problem vid eliminering av bakterier på somliga platser, t.ex. utsidan av rör, gummislangar och tak. På dessa ställen sitter mikroberna ytterst hårt fast i underlaget och har en naturlig resistens eller har utvecklat en högre motståndskraft mot desinficeringsmedel. Resultaten visade att ozoneringen hade en liten effekt på ytfästa mikrober, speciellt då dessa var intorkade på ytorna. Dimdesinficeringsanläggningens effektivitet bör mätas med jämna mellanrum så att man kan vara säker på att optimal desinficering uppnås.

- Resultaten av en förfrågning i norska mejerier visade att användningen av desinficerande fotbad är vanlig där. Målet var att kombinera praktisk information med laboratoriexperiment för att ta fram råd för hur hygienen i fotbaden kan upprätthållas. Detta var nödvändigt, eftersom man kunde påvisa att mikroberna kan överleva och växa i fotbad. Den norska rapporten initierade en undersökning på Arla, där den mikrobiologiska statusen i fotbaden undersöktes. Mikrobiologisk förorening kunde konstateras i alla testade fotbad, t.o.m då hypokloritkoncentratinen var korrekt eller högre än anbefallt. På basen av undersökningen anbefalls man numera att byta skor då man går in i ett område med högre hygien i stället för att använda desinficerande fotbad.

- Livscykelanalys (LCA) av olika CIP-metoder omfattande standard-CIP med lut, syra och hett vatten samt enzymatisk rengöring följd av syrabehandling och kemisk desinficering gjordes på TINE. LCA-analysen omfattade alla miljö-aspekter inkl. transport och utsläppseffekter. I LCA-analysen utvärderades alla potentiella miljöeffekter för varje utsläpp enligt ’worst-case’ principen. LCA bidrar med nödvändig information för att på bästa sätt kunna välja lämplig CIP-metod, trots att den är baserad på olika antaganden. I denna studie blev den enzymbaserade rengöringen utvärderad som det miljövänligaste alternativet huvudsakligen på grund av den låga koncentrationen och den låga temperaturen.

- Målet med projektet om rengöring av yoghurtfermenteringslinjen var att isolera fördärvande mikrober ss. termofila bakterier från prosessen och att finna

lämpliga rengöringsmedel för ytor nedsmutsade med yoghurt. Olika kombinationer av rengöringsmedel testades i pilotskala användande mikrober isolerade ur yoghurtprosesslinjen samt vidbränd yoghurtmjölk på rostfria stålytor. Bästa rengöringseffekt på ytor med vidbränd yoghurtmjölk erhölls med en tvåstegs-rengöring med lut innehållande kelat.

- Testing av rengöringen av ostformer gjordes både i pilot- och processkala. Strukturen av plast ostformar är komplex med långa, trånga, konformade kanaler. Ultraljud visade sig vara bra för rengöring av dessa ostformar. Ostformarnas hygien efter rengöring i pilot- och processkala utvärderades med olika metoder och dipslide-tekniken var den mest praktiska metoden för mätning av mikrobiologisk förorening. I industriell skala visade sig mätning av pH vara den bästa metoden för att bestämma hur rengöringen fungerade. Metoder baserade på det kemiska syre behovet (COD) samt EDTA-titrering visade sig båda vara använbara vid bestämning av den organiska belastning i rengöringsvattnet.

- I försöken att utveckla nya miljövänliga rengöringsmedel innehållande enzymer testades olika proteinaser (t.ex. kryotin och kymotrypsin från torsk), vilka renats på Islands universitet. En metod baserad på fluorometriska, kolorimetriska och turbidometriska mätningar användes gör att utvärdera effektiviteten av de enzymatiska rengörings-medlen på *Bacillus* biofilmer. Metoden baserad på fluorogeniska redoxindikatorer (t.ex. resazurin) användes för att utvärdera effekten av enzymatisk rengöring av biofilmer med mjölksyrabakterier, *Escherichia coli* och *Pseudomonas aeruginosa*. Proteinas proven avlägsnade biofilmen då mjölk inte fanns på ytorna.

- Evaluering av skjuvspänningens effekt (0.024–0.53 m/s) på desinficerings-medlens förmåga att lösgöra *P. aeruginosa* biofilm gjordes i en koncentrisk cylinder reaktor (CCR). Resultaten påvisade att CCR kan användas vid bedömning av olika desinficeringsmedels rengöringseffekt. Vid mätning av desinficeringseffekten och ytors sterilitet kan impedansmetoden användas, eftersom några få, adapterade, snabbväxande celler liksom stora mängder av kemiskt exponerade celler kan förändra näringsbuljongens konduktivitet och kapasitans. Resultaten visade att livskraften och totala antalet celler kan bestämmas med hjälp av infärgning av ytor. Odling var inte lämplig för

mätning av väletablerad biofilm, eftersom dessa celler ofta är hårt fästade vid ytorna och därför inte kan lösgöras genom svabbning.

- Upptagning av 1-*N*-fenylnaftylamin (NPN) kan mätas spektrofotometriskt och användas för uppskattning av permeabilisering av både Gram-negativa och Gram-positiva celler. Gram-positiva celler har inte ett skyddande yttre cellmembran och NPN har i dem fri passage till det inre membranet. NPN-resultaten av cellers permeabilisering med kelaterande ämnen och polyetylenimin (PEI) visade att cellerna är olika känsliga för olika ämnen. Resultaten visade också att citronsyra är en effektiv permeabilisator medan natriumcitrat inte fungerade som permeablisator.

- Parametrar i hydrogelmetoden, dvs. en metod baserad på konstruerad biofilm i poloxamer, optimerades för testing av olika desinficeringsmedel. Resultaten visade att en inkubering på 5 h av 100 µl hydrogel droppar ympade med teststammar var det lämpligaste testmaterialet. Effekten av desinficeringsmedel på biofilmbakterier kan optimeras med denna metod. Resultaten visade också att de Gram-negativa bakterierna är mera motståndskraftiga mot desinficeringsmedel än Gram-positiva är. Det väteperoxidbaserade medlet visade sig vara det mest effektiva mot de testade mikroberna.

Utveckling av detektions- och identifieringsmetoder för bestämning av mikrobiologiska föroreningar på prosessytor, i råvaror, i luft, på förpackningsmaterial och i slutprodukter bör få en fortsättning. Samarbetet inom mejerihygien fortsätter nu i ett nätverksprojekt ”Dairynet – Hygiene control in dairy environment” som delvis finansieras av Nordisk Industrifond (P00027).

SAMMANFATTNING

RENGÖRING AV SLUTNA SYSTEM

Testningen av ozon i CIP-rengöring och -desinficering gav resultat som påvisade endast en liten minskning av mikrober. Den huvudsakliga orsaken för detta är antagligen att man inte lyckades uppnå en effektiv, tillräckligt hög, koncentration av ozon i CIP-systemet.

Även om ozonet i sig är ett effektivt medel vid desinficering finns det fortfarande problem med att uppnå tillräckligt höga koncentrationer i CIP-systemet. CIP-systemet i detta försöket var placerat för långt från ozon-generatorn. Ozonet bröts ner när det var i kontakt med luft och därmed minskade dess effekt. För att man skall kunna använda ozon bör man först få fram riktiga, tekniska lösningarna.

JÄMFÖRELSE AV TESTMETODER FÖR UNDERSÖKNING AV DESINFICERINGSMEDELS EFFEKTIVITET

Bakteriologiska suspensionstester kan inte användas för jämförelse av kommersiella desinficeringsmedel eller känsliga bakterier, dessa metoder bör nämigen ge en hög avdödning för att de överhuvudtaget påvisar någon desinficeringseffekt. Resultat av metoder som grundar sig på exponering av biofilmbakterier och av bakterier i konstruerad biofilm (hydrogel) i desinficeringsmedel gav information både om desinficeringsmedlens avdödande effekt och om samverkan mellan desinficeringsmedlet och biofilmen.

TESTING AV LUFT DESINFEKTION I INDUSTRIELL SKALA

I de utförda testerna kunde man märka skillnader mellan de olika desinfektionsmetoderna och också mellan de olika kemiska komponenterna. Ozon var ineffektivt mot stressade mikrobceller, det hade bättre effekt på utväxta celler. I det fall att mikroberna var livskraftigt växande var det rätt enkelt att avdöda cellerna. Genom att optimera detta steg i metoden kan ozonet göras mera effektivt. Resultaten visade också att det var viktigt att använda en tillräckligt hög koncentration och tillräckligt lång kontakttid.

Ett desinficeringsmedel innehållande väteperoxid och perättiksyra var mera effektivt än det tensid-baserade medlet vid dimning. Nya medel bör testas under kontrollerade förhållanden med ytprover. Då ytprov utförs är det mycket viktigt att känna till hur mikroberna uppför sig i olika förhållanden.

YTDESINFICERING I INDUSTRIELL SKALA

Resultaten av de utförda proven visade att ozonet inte var effektivt vid den utförda ytdesinficeringen. I det fall att utrustningens kapacitet är tillräcklig och ytorna är fria från organisk smuts kan dimdesinficering användas för avdödning av bakterier på ytor. Det anbefalles att effektiviteten av desinficeringen övervakas i praktiska applikationer och att man då använder sig av flera provställen per mätning.

RESISTENSFENOMEN BASERADE PÅ DESINFICERING

Sporer och somliga resistenta vegetativa bakterier kan överleva desinficeringen eftersom de byggt upp ett visst motstånd mot desinficeringsmedlet. Somliga bakterier innehar en naturlig resistens, medan andra vänjer sig vid det använda desinficeringsmedlet. Man märkte också att sporer eller biofilmbildande bakterier inte alltid försvinner vid vanlig standard rengöring eller desinficering.

UTVÄRDERING AV OSTFORMARS RENGÖRINGSPROCEDURER

Ostformarna rengjordes med olika rengöringsmedel och -metoder och ytorna analyserades därefter med olika metoder. Ingen av testmetoderna (protein-kit Check Pro, ATP, svabbning, sköljning, UV-strålning, DEM och TTC-agar) fungerade tillfredsställande vid detektering av smuts. TTC-agarmetoden och sköljning fungerade bäst vid mätning av mikrobiell tillväxt. Dessa metoder gav liknande och reproducerbara resultat. I pilotskaleförsöken gav kontaktagarmetoden de säkraste resultaten. Det finns ett behov av att förbättra metoder med vilka organiska rester kan detekteras, så att rengöringseffekten kan utvärderas. I fullskaleförsöken visade de mikrobiologiska mätningarna att sköljvattnet var kontaminerat med ett högt antal bakterier. Metoden kan alltså också användas för

att utvärdera kvaliteten i tillförselsystem ss. kvaliteten på ånga, vatten och kemikalier.

UTVÄRDERING AV MILJÖEFFEKTEN

Huvudmålet med rengöring och desinficering i livsmedelsindustrin är att avlägsna smuts och dålig lukt, och att avdöda de mikrober som finns kvar efter rengöringen. Andra aspekter är ekonomi, miljöeffekt, säkerhet och korrosion. Livscykelanalysen (LCA) är en användbar metod då man skall kontrollera den totala effekten av olika CIP-metoder på miljön, från råvara till avloppshantering. Svagheter i LCA är osäkerheten i värdering och karakterisering av miljömässig påverkan av enskilda kemikalier ss. fosfater och tensider.

En praktisk tillämpning för evaluering av hur bra rengöringen fungerar finns beskriven i kapitel 4.2. Följande punkter i proceduren föreslås: insamling av skriftlig information, granskning av leverantörer, utvärdering av de funktionella egenskaperna, praktisk evaluering samt en slutlig utvärdering baserad på alla erhållna egenskaper.

PROSJEKTSAMMENDRAG (NORSK)

Forskningsarbeidet i prosjekt P96049 i det andre NORDFOOD programmet ble utført ved de nordiske forskningsinstituttene VTT Biotecnology, MATFORSK og SIK samt ved universitetene i Helsinki og Reykjavik fra april 1997 til Januar 2000. Meieriene som var involvert i prosjektet var Valio Ltd fra Finland, Arla fra Sverige og TINE fra Norge. I tillegg deltok det teknokjemiske firmaet Suomen Unilever Oy DiversyLever fra Finland. Dr. Gun Wirtanen, VTT Biotechnology koordinerte prosjektet. Eksperimentene fokuserte på måle metoder i renhold av åpne og lukkede systemer, for eksempel tåkelegging, ozonering, fotbad hygiene, vask av osteformer og yoghurt linjer, utvikling av metoder for å teste effekten av desinfeksjonsmidler, mikrobielle resistens fenomener, livs-syklus-analyser (LCA) og evalueringsprosedyrer for funksjonaliteten av vaskeprosedyrer. Nye hygiene løsninger basert på resultatene i prosjektet har blitt implementert i meieriene. Funnene kan oppsummeres som følgende:

- Hovedfokus i det svenske prosjektet var å utvikle og evaluere praktiske metoder for å måle effektivitet av vask og desinfeksjon. Metodene skulle kunne benyttes på utstyrsoverflater brukt ved produksjon av ulike meieriprodukter. Analysemetodene utvasking og TTC fungerte bra for å teste rengjøring av oste former av plastikk. En 5-trinns metode for å evaluere vaske- og desinfeksjonsløsninger ble utviklet ved Arla Foods.

- *Bacillus cereus* er en viktig målorganisme ved desinfeksjon av utstyr for produksjon av konsumentmelk i de nordiske landene. Total antall *Bacillus* sporer på faste overflater er en nyttig indikator for å måle effekten av renholdet. Forbedrede deteksjonsmetoder for sporer som er festet til overflater kan være basert på fluorescerende fargestoffer, indirekte immunofluorescens og kvarts krystall mikrobalanse teknikker.

- Mer miljøvennlige vaske prosedyrer ved bruk av ozonert vann eller enzym baserte vaskemidler ble utprøvd i et CIP-system. Ved disse metodene kan man spare energikostnader i tillegg til mindre forurensing av miljø. Lovende resultater med 90.0–99.9% reduksjon i sporetall ble oppnådd ved en ozonkonsentrasjon på 0.1–0.3 ppm.

- Tåkelegging med desinfeksjonsmiddel og ozonering av luft er potensielle metoder for desinfeksjon av luft og overflater. Det var imidlertid problemer med eliminere bakterier på visse områder i rommet, slik som utsiden av rør, gummislanger og tak. På disse områdene sitter mikrobene muligens fast i underlaget og har er naturlig resistens eller utvikler høyere resistens mot desinfeksjonsmidler. Resultatene vist at ozonering hadde liten effekt på mikrober på overflater, spesielt når disse er tørket inn. Ytelsen til tåkeleggings-utstyret må måles jevnlig for å sikre optimal desinfeksjon

- Resultatene av en spørreundersøkelse viste at desinfiserende fotbad er mye brukt i norske meierier. Ved å kombinere praktisk informasjon med laboratorieeksperimenter ble det laget retningslinjer for vedlikehold av fotbad. Dette var nødvendig, fordi det ble påvist at mikroorganismer kan overleve og vokse i fotbad. Den norske rapporten initierte en undersøkelse på Arla der den mikrobielle statusen til fotbad ble undersøkt. Mikrobiell forurensning ble funnet i alle fotbad testet, til og med når hypokloritt konsentrasjonen var korrekt eller over det anbefalte nivået. På grunnlag av undersøkelsene ble det anbefalt å skifte fottøy når man går inn i et område med høy hygiene fremfor å bruke desinfiserende fotbad.

- Livs-syklus-analyse (LCA) på ulike CIP-metoder inkludert standart CIP med lut fase, syre fase og hettvann og enzymatisk vask fulgt av syre behandling og kjemisk desinfeksjon ble utført ved TINE. LCA analysen dekket alle miljøaspekter inkludert transport og effekter på utslipp. I LCA blir alle potensielle miljøeffekter til hvert utslipp vurdert ut fra 'worst-case' prinsippet. LCA bidrar med nødvendig informasjon for å velge beste CIP-metode, selv om en rekke antagelser og forbehold må brukes. I denne studien ble enzym basert vask vurdert som mest miljøvennlig, hovedsakelig på grunn av lave konsentrasjoner og lav temperatur.

- Målet med prosjektet på renhold i yoghurt fermenteringslinjer var å isolere forringelsesmikrober og termofile bakterier fra prosesslinjen og å finne passende vaskemidler for overflater tilsmusset med yoghurt. Ulike kombinasjoner av vaskemidler ble testet ut i pilotskala ved bruk av isolater fra yoghurt og prosesslinje som var påbrent rustfritt stål. Best vaske-effekt på overflater med påbrent melk ble oppnådd ved en to-fase prosess med chelator-basert lut.

- Det ble utført uttesting av vask av osteformer i pilot- og full skala forsøk. Strukturen til osteformer i plast er kompleks, med lange, trange, koniske kanaler. Ultralyd viste seg å være en god metode for å vaske osteformer. Renheten til osteformene etter vask i pilotskala og under prosessering ble målt ved ulike metoder og dipslide teknikken var den mest praktiske metoden for å finne mikrobiell forurensing. I industriell skala var måling av pH beste metode for å bestemme om vaskeprosedyren fungerte. Kjemisk oksygen behov (COD) metoden og EDTA målinger var nyttige for å bestemme organisk belastning av vaskevann.

- I forsøk for å utvikle nye miljøvennlige vaskemidler basert på enzymer ble det renset proteinase prøver (for eksempel cryotin fra torsk, trypsin fra antarktisk krill og chymotrypsin fra torsk) på Universitetet på Island. En mikrotiter plate metode med fluorometriske, kolorimetriske og turbidometriske målinger av effektiviteten av de enzymatiske vaskemidlene på *Bacillus* biofilmer ble utviklet. En metode basert på fluorogeniske redoks indikatorer (for eksempel resazurin) ble brukt for å evaluere effekt av enzymvask av biofilmer med melkesyrebakterier, *Escherichia coli,* og *Pseudomonas aeruginosa*. Proteinase prøvene fjernet biofilmer i fravær av melk.

- For å evaluere effekten av skjærkrefter (0.024–0.53 m/s) av desinfeksjons-løsninger på løsrivelse av *P. aeruginosa* biofilmer ble det brukt en konsentrisk sylinder reaktor (CCR). Resultatene viste av CCR kan brukes for å skille mellom desinfeksjons- og vaskeeffekt for ulike desinfeksjonsmidler. I måling av effekten av desinfeksjonsmidler kan måling av impedans brukes for å evaluere overflate sterilitet fordi få, adapterte, hurtigvoksende celler på samme måte som store mengder kjemisk eksponerte celler kan forandre konduktiviteten eller kapasitansen til væsken. Viabilitet og totaltall celler kunne bestemmes ved hjelp av farge prosedyrer. Oppdyrking var ikke en passende metode for å måle veletablerte biofilmer på overflater fordi cellene ofte satt så godt festet til overflaten at man ikke fikk dem løs med svabring.

- 1-*N*-phenyl-naphtylamine (NPN) opptak kan måles spektrofotometrisk og brukes for å evaluere permeabilisering av både Gram-negative og Gram-positive celler. Gram-positive celler har ikke en beskyttende ytre cellemembran og NPN har fri tilgang til den indre membranen. Resultatene av uttesting av NPN-metoden for å se på permeabilisering av bakterier med chelatorer og PEI

viste at sensitiviteten for permeabiliserende stoffer varierer meget mellom arter. Resultatene viste også at sitronsyre var en effektiv permeabilisator mens natrium sitrat virket dårligere.

- Parametere i metoden basert på hydrogeler (for eksempel biofilm konstrukter av poloxamer) ble optimalisert ved bruk av ulike desinfeksjonsmidler. Resultatene viste at 5 timer inkubering av 100 µl inokulerte dråper var beste test matriks. Effekten av desinfeksjonsmidler på biofilm bakterier kan optimaliseres ved denne metoden. Resultatene viste at Gram-negative bakterier er mer resistente mot desinfeksjon enn Gram-positive. Middelet basert på hydrogen peroksid viste seg å være det mest effektive mot mikrobene som ble testet.

Utvikling av deteksjons- og identifiseringsmetoder for å bestemme mikrobiell forurensing på prosess utstyr, råvarer, luft, pakkematerialer og i sluttprodukter bør fortsette. Samarbeidet innen meieri hygiene vil fortsette i et nettverk prosjekt ”Dairynet – Hygiene control in dairy environment” finansiert av Nordisk Industrifond (P00027).

KONKLUSJONER

RENGJØRING AV LUKKEDE SYSTEMER

Testene for bruk av ozon ved CIP-rengjøring og desinfeksjon ga dårlige resultater mht reduksjon av mikrober. Hovedgrunnen for dette er antakelig at man mislyktes i å oppnå en effektiv ozon konsentrasjon i CIP-systemet.

Selv om ozon er betraktet som et effektivt middel for desinfeksjon er det fortsatt problemer med å få til konsentrasjoner som er høye nok til CIP-systemet. CIP-systemet i dette forsøket var plassert for langt unna ozon-generatoren. Ozonen ble dekomponert når den kom i kontakt med luft og dermed ble effektiviteten av ozonet redusert. For at man skal kunne bruke ozon må de tekniske løsningene på plass først.

SAMMENLIKNING AV TESTMETODER FOR EFFEKTIVITET MTP DESINFEKSJON

Bakteriologiske suspensjonstester kan ikke bli brukt for å sammenlikne kommersielle desinfeksjonsmidler eller sensitive bakteriearter. Metoder som baserer seg på å eksponere bakterier dyrket som biofilm og bakterier i biofilmkonstrukter for desinfeksjonsmidler ga sammenliknbare resultater og informasjon om den bakteriedrepende effekten og interaksjoner mellom desinfeksjonsmidlene og biofilm-matriksen.

TESTING AV LUFT-DESINFEKSJON I INDUSTRIELL MÅLESTOKK

I de utførte testene er det forskjeller mellom de ulike desinfeksjonsmetodene og også mellom de ulike kjemiske komponentene. Ozon viste seg å ikke være så effektivt for stressede mikrober som det var på bakterier i vekst. Der mikrobene var i en levedyktig og voksende fase ble de drept ganske enkelt. Bedre utnyttelse /optimalisering av denne metoden kan gjøre ozon mer effektivt. Testene viste også at det var viktig med høy konsentrasjon og lang nok kontakttid.

Desinfeksjonsmidler med hydrogenperoksyd og pereddiksyre var mer effektive enn tensid-baserte midler for tåkelegging. Det er behov for å teste nye midler under kontrollerte betingelser. Disse testene bør baseres på overflatetesting. Ved utførelse av overflatetester er det veldig viktig å kjenne mikrobenes oppførsel under de ulike omgivelser og situasjoner.

OVERFLATEDESINFEKSJON I INDUSTRIELL SKALA

Effektiviteten av overflatedesinfeksjon med ozon er ikke god. Dersom kapasiteten på utstyret er tilstrekkelig og overflatene rene kan tåkedesinfeksjon brukes til å fjerne bakterier fra overflater. Det er anbefalt at effektiviteten av praktisk desinfeksjon skal overvåkes jevnlig ved bruk av mange nok prøveuttakssteder.

Resistensfenomener grunnet desinfeksjon

Sporer og enkelte resistente vegetative bakterier kan overleve desinfeksjon fordi de bygger opp resistens mot desinfeksjonsmidler. Noen bakterier er naturlig resistente, mens andre tilpasser det desinfeksjonsmidlet som brukes. Det ble også funnet at sporer eller biofilmdannende bakterier ikke forsvinner under standard rengjørings- eller desinfeksjonsprosedyrer.

Evaluering av ostformsvask

Ostformer ble vasket med ulike midler og metoder og deretter analysert vha ulike metoder. Ingen av testmetodene (protein-kit Check Pro, ATP, svabring, skylling, UV-bestråling, DEM og TTC-agar) fungerte bra for å detektere smuss. TTC-agaren og skylling var de metodene som virket best for deteksjon av mikrobiell vekst. Disse metodene ga sammenliknbare og reproduserbare resultater. I pilot-forsøkene ga kontakt-agaren de sikreste resultatene. Det er behov for forbedring av metoder der nivået av organiske rester kan detekteres, slik at vaske-effekt kan evalueres. I full-skala forsøket viste de mikrobielle testene at rensevannet var kontaminert med et høyt antall bakterier. Metoden kan altså også brukes for å vurdere kvaliteten på vann.

Miljøaspekt

Hovedmålet med vask og desinfeksjon i matvareindustrien er å fjerne smuss og dårlig lukt, og å drepe de mikrobene som fortsatt måtte være tilstede etter vask. Andre aspekter er økonomi, miljøeffekt, sikkerhet og rust. Livssyklus-analyse er en nyttig metode dersom man skal se på total innvirking på miljø for ulike CIP-metoder, fra råvare til avløpshåndtering. Svakheten med LCA i denne sammenhengen er usikkerheten i verdier og karakterisering av miljømessig påvirkning for enkelte kjemikalier, som f eks fosfater og tensider.

En praktisk vinkling for å evaluere funksjonaliteten av vasking er beskrevet i punkt 4.2. Følgende punkter er foreslått; innsamling av skriftlig informasjon, gjennomgang av leverandør, bestemmelse av funksjonelle egenskaper, praktisk evaluering og endelig bedømmelse.

TIIVISTELMÄ JA YHTEENVETO (SUOMENKIELINEN)

Tutkimustyö NORDFOOD II -ohjelmaan kuuluvassa projektissa P96049 tehtiin pohjoismaisissa tutkimuslaitoksissa; VTT Biotekniikassa, MATFORSK:issa ja SIK:ssä sekä Helsingin ja Reykjavikin yliopistoissa huhtikuun 1997 ja tammikuun 2000 välisenä aikana. Projektissa oli mukana kolme meijeriä: Valio Suomesta, Arla Ruotsista ja TINE Norjasta sekä teknokemian yritys Suomen Unilever Oy DiverseyLever Suomesta. Gun Wirtanen VTT Biotekniikasta toimi projektin koordinaattorina. Nordic Industrial Fundin yhdyshenkilöinä toimivat Maija Uusisuo ja Oddur Gunnarsson. Tutkimukset keskittyivät avointen ja suljettujen järjestelmien puhdistusmenetelmiin liittyviin aiheisiin, kuten sumutusdesinfiointiin, otsonointiin, jalkineiden desinfiointialtaiden hygieniaan, juustomuottien ja jogurttiprosessin pastöörien puhdistamiseen sekä desinfiointiaineiden tehokkuuden testausmenetelmien kehittämiseen, mikrobien desinfiointiaineresistenttisyyden määrittämiseen, life cycle assessment (LCA) -elinkaarianalyysin laadintaan ja puhdistusohjelmien toimivuuden arviointiin. Saatujen tulosten perusteella kehitettyjä hygieniaa parantavia toimintatapoja on otettu käyttöön meijereissä. Yhteenvetona projektin tutkimustuloksista voidaan mainita seuraavaa:

- Ruotsissa tärkeimpänä tutkimusaiheena oli kehittää ja arvioida käytännöllisiä puhdistus- ja desinfiointitehokkuuden määritysmenetelmiä. Käytettävien menetelmien on sovelluttava erityyppisten meijerituotteiden valmistukseen käytettävien laitteiden pinnoilta tehtäviin määrityksiin. Käytetyt analysointimenetelmät, viljely huuhtelemalla otetusta näytteestä ja kontaktiviljely TTC-väriainetta sisältävällä agarilla, soveltuivat hyvin muovisten juustomuottien puhdistuvuuden testaamiseen. Osana tutkimusta valmisteltiin Arla Foodsissa 5-vaiheinen menetelmä puhdistuksen toimivuuden arvioimiseksi.

- Tuotettaessa pohjoismaisille kuluttajille maitotuotteita on *Bacillus cereus* -bakteerimäärien seuranta tärkeässä osassa. Pinnalle jääneiden *Bacillus*-itiöiden määrä on käyttökelpoinen indikaattori määritettäessä puhdistuksen ja desinfioinnin tehokkuutta. Pintaan kiinnittyneiden itiöiden määrittämiseen voidaan käyttää uusia menetelmiä, jotka perustuvat fluoresoivien väriaineiden käyttöön, epäsuoraan vasta-ainefluoresenssiin ja kvartsikristallimikrotasapainotekniikkaan.

- Projektissa tutkittiin ympäristöystävällisempiä puhdistusmenetelmiä, jotka perustuvat otsonoidun veden ja entsyymipohjaisten pesuaineiden käyttöön CIP-pesuissa. Energiasäästöjä saavutettiin käyttämällä alhaisempia pesulämpötiloja. Käytettäessä otsonikonsentraatioita 0,1–0,3 ppm saatiin lupaavia tuloksia, joiden mukaan itiömäärät vähenivät otsonoinnilla 1–3 logaritmiyksikköä.

- Sumutusdesinfiointi erityyppisillä desinfiointiaineilla, otsoni mukaan luettuna, on potentiaalinen menetelmä tuotanto- ja varastointitilojen desinfiointiin. Putkilinjojen ulkopinnat, muoviletkut, katot ja muut hankalasti tavoitettavat paikat ovat kuitenkin edelleen ongelma-alueita. Näille ongelmapinnoille voi kiinnittyä mikrobeja, jotka sopeutuvat desinfiointiaineeseen muodostaen resistenttikantoja, sillä näillä raja-alueilla desinfiointiaineen konsentraatiot ovat mahdollisesti laimeampia kuin pääasiallisessa pesukohteessa. Tulosten mukaan otsoni ei tehoa niin hyvin kuivassa ympäristössä oleviin mikrobeihin kuin kosteassa ympäristössä oleviin mikrobeihin. Sumutusyksikön toimivuutta pitää tarkkailla säännöllisesti optimaalisen desinfioinnin varmistamiseksi.

- Kyselyn perusteella jalkineiden desinfiointialtaat ovat laajassa käytössä norjalaisissa meijereissä. Tarkoituksena oli laatia jalkineiden desinfiointialtaiden hoito-ohjeistus käytännön kokemuksista ja laboratoriotutkimuksista saatujen tietojen perusteella. Hoito-ohjeita selvästikin tarvitaan, sillä on todettu, että jalkineiden desinfiointialtaiden käytön seurauksena muodostuu resistenttejä mikrobikantoja, jotka lisääntyvät ja leviävät tehdasympäristöön. Norjalainen raportti sai alkunsa Arla Foodsissa tehdyistä jalkineiden desinfiointialtaiden mikrobitilanteen kartoituksesta. Mikrobeja löydettiin kaikista tutkituista jalkineiden desinfiointialtaista, vaikka käytetyt natriumhydroksidikonsentraatiot olivat yleisesti ottaen suositellulla tasolla tai sen yli. Saatujen tulosten perusteella suositellaan jalkineiden vaihtoa siirryttäessä korkean hygienian alueelle jalkineiden desinfiointialtaiden käytön sijasta.

- TINEssä tehtiin LCA-elinkaarianalyysi erilaisista CIP-menetelmistä, joita olivat normaali CIP-menetelmä lipeä-, happo- ja lämpödesinfiointikäsittelyillä sekä CIP-menetelmä entsymaattisella pesu-, happo- ja kemikaalikäsittelyllä. LCA kattoi kaikki ympäristönäkökulmat mukaan lukien kuljetukset ja jätevedenkäsittelyt. LCA-elinkaarianalyysissä jokaisen vaiheen kaikki potentiaaliset ympäristövaikutukset huomioitiin ”pahin mahdollinen tapaus” -periaatteella. LCA-elinkaarianalyysistä saatiin parhaan CIP-menetelmän

valintaan tarvittavat tiedot, vaikkakin arviossa oli tehty useita oletuksia ja rajoituksia. Tämän tutkimuksen mukaan entsyymipohjainen CIP osoittautui parhaaksi menetelmäksi, koska siinä käytetyt konsentraatiot olivat pieniä ja lämpötilat alhaisia.

- Jogurtin kypsytyslinjan puhdistuvuustutkimusten tarkoituksena oli eristää jogurttiprosessista haitallisia pilaajamikrobeja, mahdollisesti myös lämpökestoisia bakteereita, ja löytää sopivat pesuaineet jogurttisten prosessipintojen puhdistukseen. Pilottimittakaavan pesukokeissa prosessista eristettyjä haittamikrobeja sisältävää jogurttimaitoa poltettiin ruostumattomalle teräspinnalle kuuman höyryn avulla ja näitä testipintoja käytettiin erilaisten pesuaineyhdistelmien vertailuun. Tutkimusten mukaan paras lopputulos pinnalle kiinnipalaneen maitolian poistoon saatiin kaksivaiheisella pesuohjelmalla, jossa käytettiin kelatointiainetta sisältävää natriumhydroksidia.

- Juustomuottien hygieniaa tutkittiin pilotti- ja prosessimittakaavoissa. Muovisten juustomuottien rakenne on puhdistuksen kannalta vaikea, koska muotissa on paljon kapeita ja pitkiä reikiä. Ultraäänipesuprosessi osoittautui tehokkaaksi menetelmäksi juustomuotin reikien puhdistukseen. Juustomuottien puhtaus pilotti- ja prosessimittakaavojen pesujen jälkeen määritettiin erityyppisillä menetelmillä, joista kontaktiagartekniikka osoittautui käytännöllisimmäksi mikrobien määritysmenetelmäksi. Teollisuusmittakaavan kokeissa pH-arvon seuraaminen osoittautui käytännölliseksi menetelmäksi pesuohjelman toimivuuden varmistuksessa. Kemiallinen hapenkulutus (COD) sekä etyleenidiamiinitetraetikkahappo (EDTA) -määritykset osoittautuivat käyttökelpoisiksi pesuveden orgaanisen likaantumisen seurantamenetelmiksi.

- Islannin yliopistossa kehiteltiin uusia ympäristöystävällisiä pesuaineita käyttämällä kaloista eristettyjä entsyymejä, kuten turskasta eristettyä kryotiinia, äyriäisplanktonista (*Euphausia superba*) eristettyä trypsiiniä ja turskasta eristettyä kymotrypsiiniä. Näiden entsyymien pesutehoa *Bacillus*-biofilmien poistoon tutkittiin mikrotiitterilevyissä sovelluksilla, jotka perustuvat fluorometriaan, kalorimetriaan ja turbidometriaan. Entsymaattisten puhdistusohjelmien arviointiin käytettiin fluorogeenisiin hapetusindikaattoreihin, kuten resatsuriiniin, perustuvia spesifisiä ja herkkiä menetelmiä. Testibiofilmeinä näissä kokeissa käytettiin maitohappobakteerien, *Escherichia colin* ja *Pseudomonas aeruginosan,* muodostamia biofilmejä. Tutkituista aineista

proteaasia sisältävät aineet olivat tehokkaita maitolikaa sisältämättömien biofilmien poistoon.

- CCR (concentric cylinder reactor, keskiösylinterireaktori) -laitteistoa käytettiin arvioitaessa pesuliuosten virtausvoimien (0,024–0,53 m/s) vaikutusta *Pseudomonas aeruginosa* -biofilmin pinnoilla pysymiseen ja irtoamiseen. Tulosten mukaan CCR:ää voidaan käyttää erottamaan erityyppisten desinfiointiaineiden biosidiset vaikutukset ja puhdistavuusvaikutukset. Arvioitaessa desinfiointiaineiden tehokkuutta pintojen desinfiointiin voidaan sovelletusti käyttää impedanssimenetelmää, koska muutamat, lujasti kiinnittyneet nopeakasvuiset solut tai kemikaaleilla käsitellyt suuret solupopulaatiot saavat aikaan muutoksia kasvatusliuoksen konduktanssi- ja kapasitanssiarvoissa. Erilaisten värjäystekniikoiden avulla voidaan määrittää pinnoilla olevien mikrobisolujen elävyys sekä kokonaislukumäärä. Viljelymenetelmä ei sovellu paksun biofilmin määrittämiseen, koska solut ovat mahdollisesti kiinnittyneet lujasti pintamateriaaliin eikä kaikkia soluja siten saada irrotettua vanutupolla määrityksiin.

- Kemikaalien tunkeutumista Gram-negatiivisten ja Gram-positiivisten bakteerien soluseinämien läpi voidaan määrittää 1-N-fenyyli-naftyyliamiinin (NPN) läpäisyyn perustuen spektrofotometrilla. Gram-positiivisilla soluilla ei ole suojaavaa ulkomembraania (outer membrane, OM), joten NPN:n pääsy sytoplasmamembraaniin on helpompaa. NPN-altistusmenetelmän tulokset kelatoivien aineiden ja PEI:n tunkeutuvuudesta bakteerisoluihin osoittivat, että ulkomembraanin läpäisyherkkyys vaihteli merkittävästi bakteerilajien välillä. Sitruunahappo osoittautui usein tehokkaaksi soluseinän läpäisijäaineeksi, kun taas natriumsitraatti oli huomattavasti heikompi aine.

- Desinfiointiaineiden tehon määrittämiseen on kehitelty hydrogeelin (poloxamerista tehty keinotekoinen biofilmirakenne) käyttöön perustuva menetelmä, jonka parametreja optimoitiin usean erityyppisen desinfiointiaineen avulla. Tulosten mukaan 5 tunnin inkubointiaika mikrobeja sisältäville 100 µl hydrogeelipisaroille oli paras testimatriisi. Tätä menetelmää voidaan käyttää määritettäessä desinfiointiaineiden tehoa biofilmissä oleviin bakteereihin. Saatujen tulosten mukaan Gram-negatiiviset bakteerit ovat vastustuskykyisempiä desinfiointiainekäsittelyille kuin Gram-positiiviset bak-

teerit. Vetyperoksidipohjaiset desinfiointiaineet tehosivat useimpiin tutkittuihin mikrobikantoihin.

Prosessilaitepinnoilta, raaka-aineista, prosessi-ilmasta, pakkausmateriaalista sekä lopputuotteesta tehtävien mikrobien määritys- ja tunnistusmenetelmiä on kehitettävä edelleen. Pohjoismaissa meijerihygieniaan liittyvä yhteistyö jatkuu Nordic Industrial Fundin rahoittamassa verkostoprojektissa ”DairyNET – Hygiene control in dairy environment” (P00027).

JOHTOPÄÄTÖKSET

SULJETTUJEN JÄRJESTELMIEN PUHDISTUS

Otsonin käytöllä ei saatu vähennettyä mikrobeja CIP-pesuissa ja -esinfioinnissa. Suurin ongelma oli saada tarpeeksi vahva otsonikonsentraatio CIP-järjestelmään. Tutkimuksessa käytetyssä koejärjestelmässä otsonigeneraattori oli liian kaukana CIP-järjestelmästä. Otsoni pääsi reagoimaan ilman kanssa, ja näin ollen otsonoinnin teho väheni. Jotta otsonoinnin käyttö onnistuisi, on ensin ratkaistava tekniset ongelmat.

DESINFIOINTIAINEIDEN TEHOKKUUDEN MÄÄRITYSMENETELMIEN VERTAILU

Bakteerisuspensiotestit eivät sovellu desinfiointiaineiden vertailuun eivätkä herkillä bakteerikannoilla tehtäviin vertailuihin. Biofilmibakteerien ja biofilminomaiseen rakenteeseen laitettujen bakteerien käyttöön perustuvat desinfiointiainetestit antoivat vertailukelpoisia tuloksia sekä bakterisidisistä tehoista että desinfiointi-aineen ja biofilmin välisistä vuorovaikutuksista.

ILMAN DESINFIOINNIN TESTAUS TEOLLISUUSMITTAKAAVASSA

Suoritetuissa tutkimuksissa oli eroja erilaisten desinfiointimenetelmien välillä sekä erilaisten kemikaalien välillä. Otsoni ei ollut niin tehokasta stressattuihin mikrobeihin kuin kasvuvaiheessa oleviin mikrobeihin. Elävät ja kasvuvaiheessa

olevat mikrobit olivat helposti tuhottavissa. Tätä vaihetta hyväksikäyttäen voitaisiin otsonointi saada tehokkaammaksi. Tutkimusten mukaan on myös tärkeää käyttää suurta konsentraatiota ja tarpeeksi pitkää vaikutusaikaa.

Sumutusdesinfiointi vetyperoksidi- ja peretikkahappopohjaisilla desinfiointiaineilla oli tehokasta. Sumutusdesinfiointi tensidipitoisilla aineilla ei ollut tehokasta. Uusien desinfiointiaineiden tehon testaukseen tarvitaan testausjärjestelmä, jonka pitäisi perustua pintatestien käyttöön. Pintatestejä tehtäessä on tärkeää tietää tutkimuksessa käytettyjen mikrobien käyttäytyminen erilaisissa ympäristöissä ja tilanteissa.

PINTADESINFIOINTI TEOLLISUUSMITTAKAAVASSA

Otsonin tehokkuus pintojen desinfiointiin ei ole hyvä. Sumutusdesinfiointia voidaan käyttää bakteerien tuhoamiseen pinnoilta, mikäli pinnat ovat puhtaita ja sumutuslaitteiston kapasiteetti on riittävä. Desinfioinnin tehokkuuden säännöllistä seurantaa todellisessa käyttöympäristössä suositellaan tehtäväksi käyttäen tarpeeksi monta näytteenottopaikkaa.

DESINFIOINNIN AIHEUTTAMA RESISTENSSI-ILMIÖ

Itiöt sekä resistentit vegetatiiviset bakteerit voivat säilyä hengissä desinfiointikäsittelystä, jos niille on kehittynyt vastustuskyky käytettävälle desinfiointiaineelle. Toiset bakteerit voivat kehittää vastustuskyvyn useita eri desinfiointiaineita vastaan, kun taas toiset bakteerit voivat olla resistenttejä pelkästään sille desinfiointiaineelle, jota on käytetty ja johon bakteerit ovat totuttautuneet. Tutkimuksissa todettiin myös, että biofilmejä muodostaneet bakteerit ja itiöt eivät tuhoutuneet normaaleilla puhdistus- ja desinfiointiohjelmilla.

JUUSTOMUOTTIEN PUHTAUDEN ARVIOINTI

Juustomuotit pestiin käyttäen erityyppisiä pesuaineita sekä erilaisia pesumenetelmiä. Pesutulosta analysoitiin seuraavilla menetelmillä: valkuaisainejäämätesti Check Pro, ATP:n määritys luminometrillä, viljely vanutupolla otetusta

näytteestä, viljely huuhtelemalla otetusta näytteestä, visuaalinen tarkastelu UV-valolla, värjätyn pinnan tutkiminen epifluoresenssimikroskoopilla (DEM) sekä kontaktiviljely TTC-väriainetta sisältävällä agarilla. Mikään käytetyistä menetelmistä ei pystynyt määrittämään pinnalla ollutta likaa hyvin. TTC-agar- ja huuhtelumenetelmä olivat luotettavimmat menetelmät mikrobikasvun määrittämiseen. Näillä menetelmillä saatiin keskenään yhteneviä ja toistettavia tuloksia. Pilottimittakaavan kokeissa saatiin luotettavimmat tulokset kontaktiagar-menetelmällä. Jotta varsinaista puhdistuksen tehokkuutta pystyttäisiin arvioimaan, on tarpeen kehittää menetelmiä orgaanisten jäämätasojen määritykseen. Teollisuusmittakaavan kokeissa mikrobiologiset testit osoittivat, että huuhteluvesi oli saastunut ja sisälsi runsaasti bakteereita. Täten todettiin, että tutkittuja menetelmiä voidaan käyttää myös arvioitaessa ylläpitojärjestelmiin kuuluvia osioita, kuten pesuaineiden tehokkuuksia.

YMPÄRISTÖVAIKUTUSTEN ARVIOINTI

Elintarviketeollisuudessa puhdistuksen ja desinfioinnin pääasiallisena tarkoituksena on lian ja likakerrostumien poisto sekä puhdistetuille pinnoille mahdollisesti jääneiden mikrobien tuhoaminen. Muita puhdistukseen liittyviä huomionarvoisia tekijöitä ovat taloudellisuus, ympäristövaikutukset, turvallisuus ja ruostuminen. Life cycle analysis – LCA eli elinkaarianalyysi (4.1) on käyttökelpoinen menetelmä arvioitaessa erityyppisten CIP-pesumenetelmien ympäristövaikutuksia ja se kattaa kaikki vaiheet raaka-aineista jätteidenkäsittelyyn. LCA:n heikkous näissä puitteissa oli epävarmuudet tiettyjen kemikaalien, kuten fosfaattien ja tensidien, ympäristövaikutuksien luonnehdinnassa ja arvioinnissa.

Kohdassa 4.2 kuvataan käytännön sovellus puhdistuksen toimivuuden arviointiin. Suositellut vaiheet ovat kirjoitetun tiedon arviointi, tavarantoimittajien auditointi, toiminnallisten ominaisuuksien arviointi ja pesutuloksen arviointi käytännön tasolla sekä ympäristövaikutukset, toimintaominaisuudet ja taloudelliset tekijät huomioiva loppupäättely.

SAMANTEKT (ÍSLENSKA)

Rannsóknarvinnan i verkefninu P96049 í öðrum hluta NORDFOOD verkefnisins var unnin af háskólum, opinberum stofnunum og fyrirtækjum á Norðurlöndunum. Aðilarnir sem um ræðir eru VTT Biotechnology, Valio Ltd, Suomen Unilever Oy DiverseyLever og Háskólinn í Helsinki (Finnlandi), Matforsk og TINE (Noregi), SIK og ARLA (Sviþjóð) og Háskóli Íslands (Íslandi). Verkefnið stóð yfir frá apríl 1997 til janúar 2000 og var dr. Gun Wirtanen frá VTT Biotechnology yfirumsjónarmaður verkefnisins. Yfirstjórn þessa verkefnis að hálfu norræna Iðnaðarsjóðsins voru Maija Uusisuo og Oddur Gunnarsson. Tilraunir sem voru framkvæmdar í verkefninu beindust einna helst að því að athuga þær aðferðir sem notaðar eru við þrif og sótthreinsun á opnum og lokuðum kerfum. Má þar t.d. nefna þokuúðun, ósoneringu, notkun sótthreinsimotta, hreinsun á gerilsneyðingartækjum, þróun á aðferðum til að meta virkni sótthreinsiefna, þolni örvera gegn sótthreinsiefnum, líftímaákvörðun (LCA) og þróun á aðferðum sem meta árangur þrifa og sótthreinsunar í mjólkursamlögum. Út frá þeim niðurstöðum fengist hafa í verkefninu hefur verið breytt um aðferðir hvað varðar hreinsunarferli í mjólkursamlögum. Helstu niðurstöður verkefnisins eru raktar hér á eftir:

- Meginverkefni í sænsku rannsóknarvinnunni var að þróa og meta hentugar aðferðir til að mæla virkni þvotta- og sótthreinsaðferða. Þessar aðferðir eiga að vera hentugar til að meta yfirborð tækja sem eru notuð til framleiðslu á margvíslegum mjólkurafurðum. Tvær aðferðir virkuðu best við sótthreinsun á ostaformum, s.k. úthreinsun (washing out) og s.k. TTC- aðferð. Báðar aðferðirnar byggjast á að mæla virkni sótthreinsunar og dauðatíðni örvera. Einn hluti í þessari vinnu var unnin af Arla Foods og fólst í því að útbúa 5 þátta aðferð til þess að meta virkni þvotta- og sótthreinsiefna.

- *Bacillus cereus* er mikilvæg baktería í sambandi við framleiðslu á neyslumjólk á Norðurlöndunum. Heildarfjöldi gróa sem finnast á vinnsluflötum eftir þvott og sótthreinsun er góður mælikvarði á virkni hreinsunarinnar. Betri greiningaraðferðir á gróum sem loða við yfirborð má fá með litarefnum og óbeinum ónæmisljósnemum.

- Umhverfisvænni aðferðir, sem byggjast á ósóneruðu vatni og efnum sem innihalda ensím sem eru notuð í lokuðum kerfum (CIP), voru rannsakaðar. Orkunotkun mætti síðan minnka með því að nota lægra hitastig. Niðurstöður sýna 1–3 Log eininga fækkun á gróum með því að nota 0.1–0.3 ppm osón styrk.

- Þokuúðun með mismunandi efnum sem innihalda osón er aðferð sem er oft notuð til þess að sótthreinsa framleiðslu- og geymslurými. Það er hins vegar erfitt að ganga úr skugga um að efnið nái til allra svæða eins og t.d. á yfirborð pípulagna, á gúmmípakkningar og á loft. Á þessum stöðum eru oft bakteríur sem eru búnar að koma sér fyrir (biofilmur) og eru orðnar þolnar gegn þeim efnum sem eru notuð. Niðurstöðurnar sýndu að osón er ekki eins virkt gegn örverum á þurrum stöðum. Einnig þarf að hafa stöðugt eftirlit með virkni þokuúðunarinnar.

- Notkun sótthreinsimotta er útbreidd í mjólkursamlögum í Noregi. Markmiðið var að tengja saman hagnýta þekkingu og niðurstöður rannsókna til þess að þróa og búa til leiðbeiningar varðandi notkun á sótthreinsimottum í matvælavinnslu. Slíkar leiðbeiningar eru augljóslega nauðsynlegar vegna þess að það er ljóst að þolnar bakteríur lifa af, vaxa og dreifast í slíkum sótthreisimottum. Norska rannsóknarskýrslan byrjar á að greina frá tilraun hjá Arla Foods þar sem örverufræðilegt ástand sótthreinsimotta var kannað. Örverumengun fannst í öllum sótthreinsimottum, jafnvel þótt klórmagnið í þeim var rétt eða jafnvel hærra en þau gildi sem teljast eðlileg notkunargildi. Niðurstöður rannsóknarinnar leiddu til að mælst var til þess að skipta um fótbúnað í stað þess að nota sótthreinsimottur þegar farið er inn á hrein vinnslusvæði.

- Líftímagreining (LCA) á mörgum CIP-aðferðum, þ.á.m. stöðluðum aðferðum með basískum fasa, sýrufasa og hitameðferð og ensímhreinsun, auk sýrumeðferðar og sótthreinsunar með efnum var framkvæmt hjá TINE. Líftímagreiningin náði yfir alla umhverfisfræðilega þætti, sem og flutning og áhrif á úrgangsvatn. Í líftímagreiningu eru öll hugsanleg umhverfisfræðileg áhrif teknin til athugunar með það í huga að um sé að ræða að það versta geti komið fyrir (worst case principle). Líftímagreiningar gefa nauðsynlegar upplýsingar til þess að velja bestu CIP-hreinsiaðferðina. Í þessari rannsókn

kom í ljós að CIP-hreinsun með ensímum gaf bestu niðurstöðuna vegna þess að ensímin eru notuð í mjög litlu magni og við lágt hitastig.

- Markmiðið með hreinlætisrannsókn á vinnslulínu við framleiðslu á jógúrt var að einangra skemmdarörverur, hugsanlega hitakærar bakteríur úr framleiðslunni og að finna hentugt hreinsiefni til þess að nota á yfirborð þeirra tækja sem notuð eru í framleiðslunni. Í pilot skala tilraunum með margvísleg sótthreinsiefni var mjólkin, sem nota átti til jógúrtframleiðslu, og bakteríur sem notaðar eru í framleiðsluferlinu fest með hitameðhöndlun á yfirborð úr ryðfríu stáli. Síðan voru gerðar tilraunir með það hvernig best væri að hreinsa efnin í burtu og sótthreinsa yfirborðið. Í ljós kom að best var að nota tveggja fasa hreinsikerfi þar sem notuð voru útfellingarefni og þvottaefni sem innihalda hydroxýl efni.

- Rannsóknirnar á hreinsun ostaforma var bæði gerð í pilot skala og í fullum skala. Byggingin á plastefninu sem ostaformin eru búin til úr er mjög flókin, með löngum þröngum kónískum göngum (við losun á mysunni). Últrahljóðbylgjur reyndist vera besta aðferðin til þess að hreinsa þessi göng. Fylgst var með hversu vel hreinsunin tókst, bæði í pilot skala og í fullum skala, með margvíslegum aðferðum. Aðferð sem kallast ”Dipslide” aðferðin reyndist vera best til þess fallin að greina örverufræðilega mengun. Í iðnaðarskala reyndist mæling á sýrustigi vera góður mælikvarði á hvort hreinsun hefði tekist. Með því að mæla COD og ethylene diamine tetracetic sýru (EDTA) var hægt að fylgjast með lífrænni mengun í hreinsivatninu.

- Við Háskóla Íslands fóru fram tilraunir til þess að þróa ný umhverfisvæn þvottaefni til að hreinsa próteinmengaða fleti (sýni). Sem dæmi um þau efni sem athuguð voru má t.d. nefna cryotín og chymotrysín úr þorski og trypsín úr uppsjávarfiskum. Þróuð var örtítrunaraðferð sem er byggð á flúoriserandi mælingum, ljósmælingum og þéttnimælingum til þess að athuga virkni þessara ensíma á *Bacillus* biofilmur. Aðferð til þess að meta sérhæfni og næmni ensíma á biofilmur annarra baktería (mjólkursýrubaktería, *Eschericia coli*, *Pseudomonas aeruginosa*) byggðist á flúoriserandi afoxunar indikatorum, eins og t.d. resazurin. Þeir próteasar sem voru prófaðir reyndust mjög vel til þess að hreinsa biofilmur sem innihéldu ekki mjólkurleifar.

- Til þess að meta áhrif eiginleika sótthreinsilausna á *P. aeruginosa* biofilmur var s.k ”concentric cylinder reactor (CCR) notaður. Niðurstöðurnar sýndu að CCR má nota til þess að greina á milli bakteríudrepandi þvottaáhrifa mismunandi sótthreinsiefna. Til þess að athuga virkni sóthreinsiefnanna má nota leiðnimælingar til þess að meta hvort yfirborð sé dauðhreinsað vegna þess að mjög fáar, vel aðlagaðar, hraðvaxandi frumur sem og mikið magn af frumum sem hafa verið hreinsaðar, breyta leiðninni í vökvanum. Með litunaraðferðum má fá fram lifandi frumur og heildarfrumufjöldann á yfirborðsflötum. Ræktun örvera er ekki nægjanlega góð aðferð til þess að mæla biofilmur sem hafa náð sér vel á strik á yfirborðsflötum vegna þess að frumurnar geta verið mjög fastar og losna ekki þó að strokusýni sé tekið.

- Mæling á 1-N-phenyl-naphthylamin (NPN) upptöku með ljósgleypnimælingu er hægt að nota til þess að meta niðurbrot frumuhimna hjá bæði Gram neikvæðum og Gram jákvæðum bakteríum. Gram jákvæðar bakteríur hafa ekki ytri himnu (LPS) og NPN á miklu auðveldara með að komast að frumuhimnunni sjálfri. Niðurstöðurnar á upptöku (gegndræpi) NPN með útfellingarefnum og PEI sýndi að mikill munur var á milli bakteríutegunda. Almennt má segja að sítrónusýra reyndist vera með mjög góða frumugegndræpi en natríum cítrat var mun veikari.

- Mælieiningar í prófi sem byggt er á s.k. ”hydrogeli” var rannsakað með því að nota mismunandi sótthreinsiefni. Niðurstöðurnar sýndu að 5 klst. ræktunartími 100 l ræktar gaf bestu niðurstöðuna. Virkni sótthreinsiefnanna á biofilmur má "optimera" með þessari aðferð. Niðurstöðurnar sýndu að Gram neikvæðar bakteríur hafa meira þol gegn sótthreinsimeðferð en Gram jákvæðar bakteríur. Sótthreinsiefni sem innihéldu vetnisperoxíð reyndust vera virk gegn flestum örverum sem voru athugaðar í þessari rannsókn.

Ljóst er að halda verður áfram að þróa aðferðir til þess að finna og greina örverufræðilega mengun á tækjum sem notuð eru í matvælavinnslu, í hráefninu sjálfu, í loftinu í vinnslunni, á þeim pakkningum sem notaðar eru og í lokaafurðunum. Samvinna á þessu sviði mun halda áfram á Norðurlöndunum í verkefninu ”DairyNET ´Hygiene control in dairy environment” sem er styrkt af Norræna Iðnaðarsjóðnum (P00027).

NIÐURSTÖÐUR

ÞVOTTUR/SÓTTHREINSUN Í LOKUÐUM KERFUM (CIP)

Þær tilraunir sem gerðar voru með notkun á ósoni til þvotta og sótthreinsunar í lokuðum kerfum reyndust ekki sem skyldi hvað varðar fækkun á örverum. Ástæðuna má trúlega rekja til þess að ekki hafi tekist að dreifa efninu í nægilegum styrkleika í lokuðu kerfi.

Þrátt fyrir að notkun ósons sé talin afar árangursrík aðferð til sótthreinsunnar þá er enn nokkuð vandamál að dreifa ozoni í nægilega háum styrkleika í lokuðum kerfum. Lokaða kerfið, sem notað var í þessari rannsókn, var of langt frá ósontækinu og þ.a.l. Spilltist ósonið þegar það komst í snertingu við loft og virkni þess minnkaði. Nauðsynlegt er því að leysa tæknileg atriði í þessu sambandi áður en hægt verður að nota óson með fullnægjandi árangri.

SAMANBURÐUR Á MISMUNANDI AÐFERÐUM VIÐ MAT Á SÓTTHREINSUN

Það er ekki hægt að nota örverudrepandi lausnarpróf (suspension test) til að bera saman áhrif mismunandi sótthreinsiefna eða við samanburð á næmum tegundum baktería. Þær aðferðir sem annars vegar byggjast á því að bera saman áhrif sótthreinsiefna á óvarðar (exposed) bakteríur sem eru ræktaðar sem biofilmur og hins vegar bakteríur verndaðar innan í biofilmu gáfu sambærilegar niðurstöður og veittu jafnframt upplýsingar um örverudrepandi áhrif sótthreinsiefna auk upplýsinga um víxlverkun sótthreinsiefnanna og grunnmassa biofilmunnar.

NOTKUN LOFTBORINNA SÓTTHREINSIEFNA Í IÐNAÐARSKALA

Í mælingum kom fram að munur var á milli mismunandi sótthreinsiaðferða en einnig kom fram munur á milli þeirra sótthreinsiefna sem notuð voru. Óson reyndist ekki vera jafn árangursríkt gegn örverum í dvala eins og gegn örverum í virkum vaxtarfasa. Í þeim tilfellum þar sem örverur voru í virkum fasa reyndist næsta auðvelt að eyða þeim og myndi hagnýting þessarar vitneskju auka árangur af notkun ósons. Tilraunir sýndu ennfremur að það var mikilvægt að nota mikinn

styrk og nægilega langan virknitíma. Þegar notuð voru sótthreinsiefni sem innihéldu vetnisperoxíð og peredikssýru þá reyndist þokuúðun skila nægilega góðum árangri, en úðun með sótthreinsiefnum sem innihéldu tensíð skilaði ófullnægjandi árangri. Nauðsynlegt er að prófa ný efni við stýrðar aðstæður. Slíkar rannsóknir þurfa að byggja á yfirborðsmælingum. Við framkvæmd yfirborðsmælinga er afar nauðsynlegt að þekkja vel viðbrögð og hegðun örvera við mismunandi aðstæður og í ólíku umhverfi.

Yfirborðssótthreinsun í iðnaðarskala

Árangur af sótthreinsun yfirborðs með ósoni skilar ekki góðum árangri. Ef geta tækis er nægjanleg og yfirborðin hrein er hægt að nota sótthreinsun með þokuúðun til að eyða bakteríum af yfirborðum. Ráðlagt er að fylgjast vel með árangri sótthreinsunnarinnar við raunaðstæður og að taka sýni á mörgum mismunandi stöðum.

Ónæmi af völdum notkunnar sótthreinsiefna

Gró og nokkrar ónæmar bakteríur geta lifað af sótthreinsun þar sem þær hafa byggt upp þol gegn sótthreinsiefnum. Sumar bakteríur hafa eðlislægt þol en aðrar hafa aðlagað sig að þeim sótthreinsiefnum sem notuð eru. Eftir því var tekið að hefðbundnar þvotta- og sótthreinsiaðferðir dugðu oft ekki til að eyða gróum og bakteríum sem höfðu myndað biofilmu.

Mat á þrifum á ostaformum

Þrif á ostaformum voru framkvæmd þar sem notast var við ýmsar tegundir þvottaefna og þvottaaðferða og árangurinn síðan metinn með mismunandi aðferðum. Engin þeirra aðferða sem notuð var (prótein-próf (Check Pro), ATP-ljósmæling, penslun, úthreinsun, UV-lýsing, DEM og TTC agar) skilaði góðum árangri við mat á óhreinindum. TTC-aðferð og s.k. úrheinsun reyndust þó skila áreiðanlegustu niðurstöðunum við greiningu á örverum. Þessar aðferðir skila niðurstöðum sem eru bæði samanburðarhæfar og hægt er að endurtaka þær aftur og aftur. Í tilraunum þar sem líkt er eftir raunverulegum aðstæðum (pilot-scale)

reyndist snertiskálaaðferðin gefa áreiðanlegustu niðurstöðurnar. Nauðsynlegt er að bæta aðferðir við greiningu leifa af lífrænum uppruna þannig að hægt sé að sannreyna áhrif þrifa til hins ýtrasta. Í tilraunum í fullum skala sýndu öruverumælingar að mikið fannst af bakteríum í því vatni sem notað var til skolunnar. Aðferðirnar má þ.a.l. einnig nota til að meta breytur í dreifikerfum eins og t.d. áhrif hreinsiefna.

MAT Á ÁHRIFUM UMHVERFISÞÁTTA

Megintilgangur þrifa og sótthreinsunar í matvælaiðnaði er að fjarlægja óhreinindi og aðskotaefni og eyða öllum örverum af yfirborði þeirra flata sem hreinsaðir eru. Önnur atriði sem þarf að huga að eru þættir eins og kostnaður, umhverfisáhrif, öryggi og tæring. Líftímagreining (4.1) er árangursrík aðferð við að skoða heilstætt umhverfisáhrif mismunandi CIP-aðferða, þar sem byrjað er á að skoða öflun hráefnis og endað á að skoða frárennsli eftir vinnslu. Það er að vísu viss veikleiki við notkun líftímagreiningar í þessu samhengi að erfitt getur reynst að meta umhverfisáhrif vissra efna eins og t.d. fosfata og tensíða.

Í kafla 4.2 er lýst hagnýtri nálgun við mat á virkni þrifa. Eftirfarandi aðgerðir eru ráðlagðar, en þær eru mat á skriflegum upplýsingum, endurskoðun birgja, mat á virkum eiginleikum, hagnýtt mat og lokaskoðun.

APPENDIX 8

CHARACTERISATION OF *SERRATIA MARCESCENS* SURVIVING IN DISINFECTING FOOTBATHS

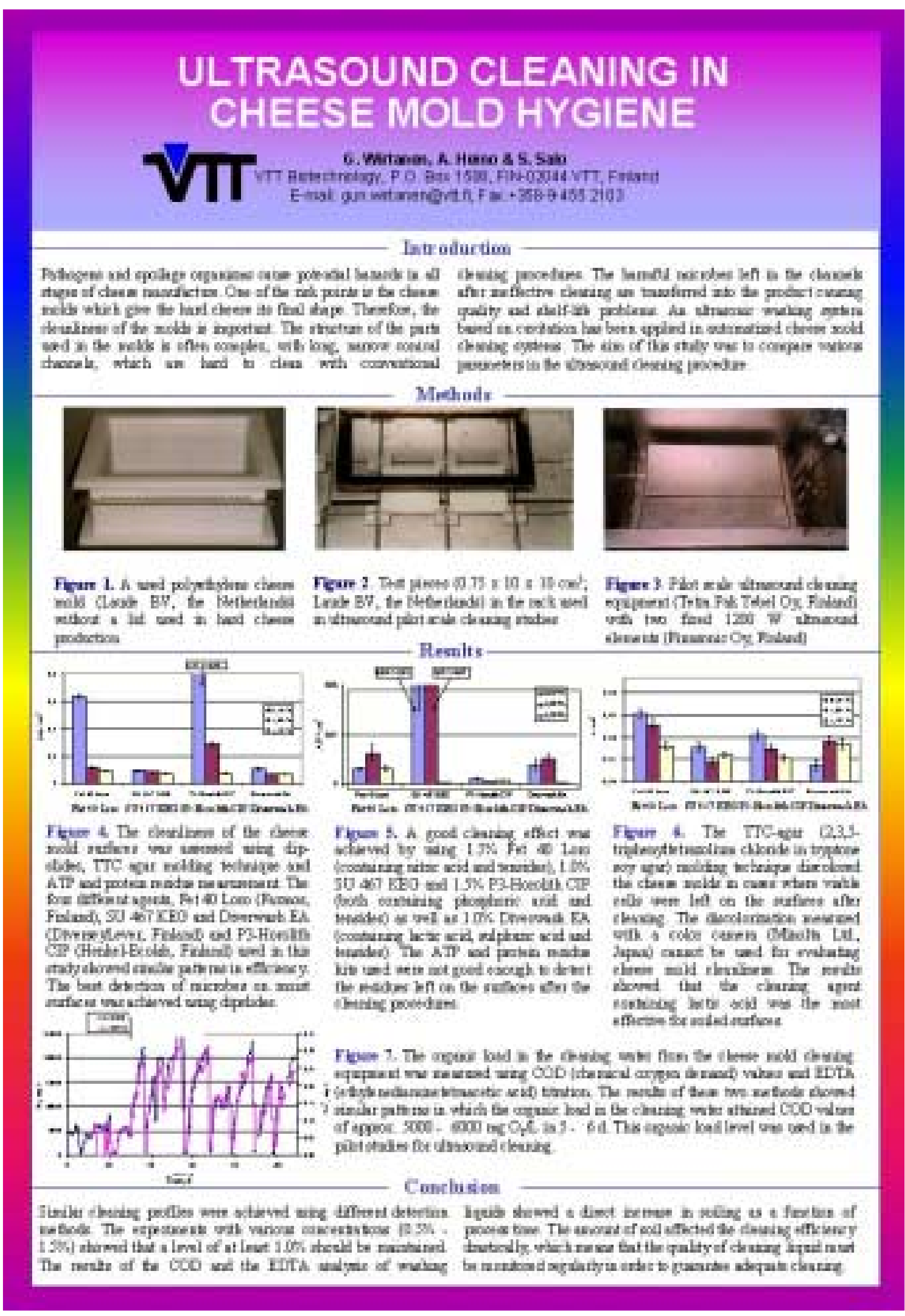
Solveig Langsrud, Trond Møretrø and Gunhild Sundheim
MATFORSK, Norwegian Food Research Institute, Oslovn. 1, Ås, Norway

Aim: To determine if disinfecting footbaths in food industry were contaminated with bacteria and characterise some of the bacteria present.

Methods and results: Bacterial strains were isolated from disinfecting footbaths containing TEGO 103G (amphoteric disinfectant) or TP-99 (alkylaminoacetate-based disinfectant) in five out of six dairy factories. Fourteen strains identified as Cedecea spp. by their fatty acid composition were further characterised. The reactions in the Rapid ID 32 E API analysis and 16S-rDNA-sequensing showed that all strains were Serratia marcescens. In contrary to Ser. marcescens ATCC 13880 the isolates from disinfecting footbaths were not killed (<5 log reduction) by the recommended in-use concentration of TEGO 103G, TEGO 51 or benzalkonium chloride. Survival and multiplication in tap water with in-use concentration of TEGO103G was demonstrated for one of the strains. All strains were killed by the in-use concentrations of commercial disinfectants based on peracetic acid, hypochlorite, quaternary ammonium compounds and alkyl aminoacetate (TP-99). There were no indications of cross-resistance between disinfectants and antibiotics.

Conclusion: Serratia marcescens may survive and multiply in disinfecting footbaths containing TEGO103G or alkylaminoacetate due to disinfectant resistance.

Significance and impact of the study: Disinfecting footbaths may act as contamination sources in food factories and they should not be used without regular hygienic monitoring.



Poster presentation Ultrasound cleaning in cheese mold hygiene based on the Pro Gradu thesis by Antti Heino and presented at the IAFP Annual meeting in August 2000.

APPENDIX 10

COMPARISON OF ULTRASOUND BASED CLEANING PROGRAMS FOR CHEESERY UTENSILS

Gun Wirtanen, Satu Salo, Antti Heino, Tapani Hattula and Tiina Mattila-Sandholm
VTT Biotechnology, Tietotie 2, Espoo, Finland

The cleaning of the plastic cheese moulds is a challenging task. The structure of the parts used in these utensils is often complex, with long, narrow conical channels, which are hard to clean with conventional cleaning procedures. Furthermore the cleaning procedure must be performed quickly, efficiently, economically and environmentally friendly without harming the surface material. If harmful microbes remain in the channels after cleaning they may be transferred into the product causing quality and shelflife problems. An ultrasonic washing system based on cavitation has been applied in automatized cheese mould cleaning systems in a Finnish cheesery. The aim of this study was to compare various parameters in the ultrasound cleaning procedure using different detection methods to optimise the washing procedure. Parameters in the test series were type and concentration of cleaning agents and ultrasound frequency. The results showed that it is important to know the principles of the measuring method used to be able to interpret the results correctly. The organic load determinations for the cleaning agent experiments were performed using the EDTA (ethylenediamine tetraacetic acid) and COD (chemical oxygen demand) methods. The organic load in the cleaning liquid affected the efficiency of the various cleaning agents used. Experiments with cleaning agent concentrations of 0.5%, 1.0% and 1.5% showed that the concentration should be maintained at least at a level of 1.0%. An increase in the ultrasound intensity from 460 W to 740 W enhanced cleaning especially in experiments using artificially aged cheese moulds.

EFFECTS OF CLEANERS ON BIOFOULED STAINLESS-STEEL SURFACES IN YOGHURT MANUFACTURING EQUIPMENT

G. Wirtanen, S. Kontulainen and S. Salo
VTT Biotechnology, P.O. Box 1500, FIN-02044 VTT, Finland
E-mail: gun.wirtanen@vtt.fi, Fax: +358-9-455 2103

Introduction

Long processing times cause fouling on equipment surfaces, especially in plate heat exchangers, e.g. in yoghurt manufacturing. The growth of harmful thermophilic bacteria in the fouled layer is referred to as biofouling. Bacteria may pass through from the surfaces of the heat exchanger to the fermentation tank. These bacteria may prolong yoghurt fermentation. Therefore, the correct processing and cleaning procedures should be specified to meet the quality claims of the final product and to keep processing costs as low as possible.
The aims of these experiments were to study the effect of harmful thermophilic bacteria in yoghurt fermentation and evaluate the cleaning efficiency of various sanitizers using biofouled stainless-steel surfaces. The bacteria used were isolated from a yoghurt processing line.

Methods

Figure 1. Milk was burned for 8 h on stainless-steel coupons (AISI 304, 2B) using the equipment shown above. Biofilms were also formed on the coupons for 4h (40 L pasteurized milk inoculated with harmful thermophilic bacteria) in the Bioreg (Tuokki Oy, Finland), where the milk (43°C) was circulated.

A

B

Figure 2. Coupons were cleaned with three different detergents, with or without acid treatment, using both A) Bioreg, marked "in tank" and B) Toreg (Tetra Pak Oy, Finland) marked "in pipeline".

Results

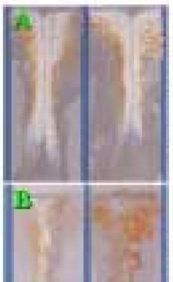


Figure 4. Stainless-steel surfaces soiled with burned yoghurt milk containing harmful thermophilic bacteria using steam heating for 8 h, A) before cleaning and B) after two phase cleaning with 0.7% sodium hydroxide (NaOH) containing 0.2% SU 560 (chelator) and 1.0% nitric acid (HNO_3).

Table 1. The test coupons were stained with acridine orange after cleaning using various procedures (reference samples were not cleaned). These coupons were examined with an epifluorescence microscope and the area covered with milk soil and microbes was determined using an image analysis system. The best cleaning result was achieved using the the two phase cleaning with 0.7% NaOH containing 0.2% SU 560 and 1.0% (HNO_3).

Cleaning agent (w/w %)	Biofouled in Bioreg (8h growth)			Soiled with burned (for 8h) yoghurt milk	
	before cleaning %	cleaned in tank %	cleaned in pipeline %	cleaned in tank %	cleaned in pipeline %
2.0 % NaOH, 1.0 % HNO_3	43.3	0.8	0.6	53.3	44.8
2.0 % NaOH	30.3	3.3	1.3	63.6	39.6
0.7 % NaOH, 0.2 % SU 560, 1.0 % HNO_3	**38.8**	**0.3**	**0.2**	**12.6**	**18.7**
0.7 % NaOH, 0.2 % SU 560	36.3	0.4	0.3	38.8	37.9
1.0 % KOH, 1.0 % HNO_3	43.3	0.6	0.3	33.8	33.3
1.0 % KOH	32.8	0.6	0.6	33.6	33.7

Conclusion

The two-phase cleaning procedure using an alkaline mixture containing chelating agents as well as nitric acid was the most efficient combination for cleaning burned milk from stainless steel. In general, the results showed that the acidic treatment enhanced the cleaning result.
The harmful thermophilic bacteria did not survive the cleaning treatments, but the remaining soil was also a risk factor offering an attachment place for new contaminants. The detachment of burned yoghurt milk is therefore a very important but difficult task to achieve without strong mechanical forces.

Poster presentation Effects of cleaners of biofouled stainless-steel surfaces in yoghurt manufacturing equipment based on the Pro Gradu thesis by S. Kontulainen.

APPENDIX 12

Abstract of the original article published in International Journal of Food Microbiology 56 (2000) pp. 81–86.

POTENTIATION OF THE LETHAL EFFECT OF PEROXYGEN ON *BACILLUS CEREUS* SPORES BY ALKALI AND ENZYME WASH

Solveig Langsrud, Birgitta Baardsen and Gunhild Sundheim
MATFORSK Norwegian Food Research Institute, Oslovn. 1, Ås, Norway
Received 5 July 1999; revised 24 September 1999; accepted 10 January 2000.
Available online 18 May 2000.

Bacillus cereus present in pipes and heat-exchangers represents a potential quality problem for dairy industry. The peroxygen-containing disinfectants investigated had only negligible sporicidal effect when applied at the recommended in-use temperature and concentration. However, cleaning agents used before disinfection potentiated their lethal activity. Pre-exposure of *B. cereus* spores to 1% sodium hydroxide at temperatures over 40°C increased the sporicidal effect of the peroxygen-containing disinfectant. The effect was dependent on the alkali concentration and the temperature. Also, a significant potentiating activity of an enzyme-based cleaning agent was obtained, but the effect was smaller than for alkali treatment. The results indicated that disinfectants based on peroxygen can be used to eliminate *B. cereus* spores at non-corrosive temperatures and concentrations if the surfaces are cleaned with alkali or enzyme-based disinfectants prior to disinfection.

Author Keywords: *Bacillus cereus*; Spores; Cleaning; Disinfection; Dairy

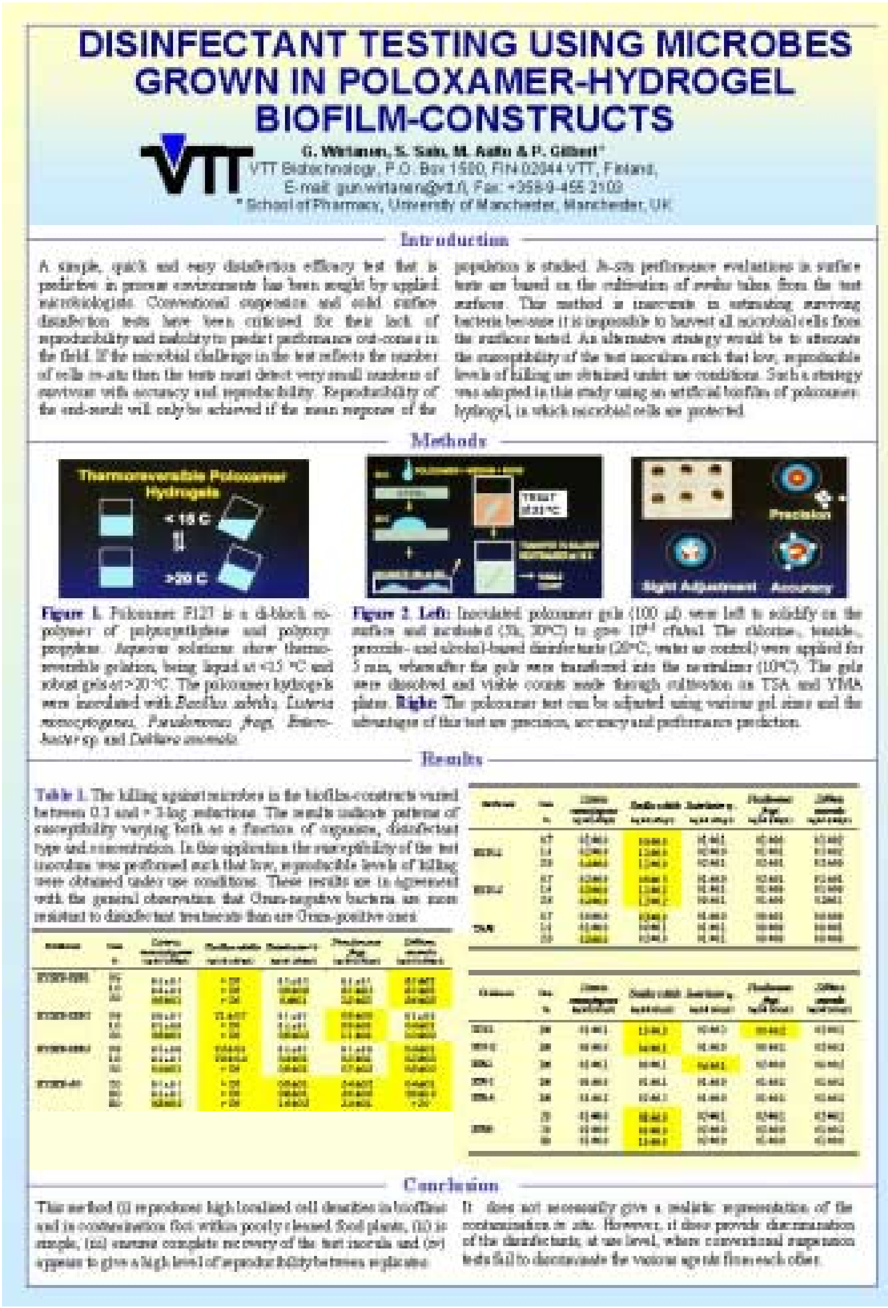
Abstract of the original article published in Journal of Industrial Microbiology 25 (2000) pp. 235–241.

INFLUENCE OF FLUID DYNAMIC FORCES UPON THE STEADY-STATE POPULATION DYNAMICS IN MICROBIAL BIOFILM COMMUNITIES

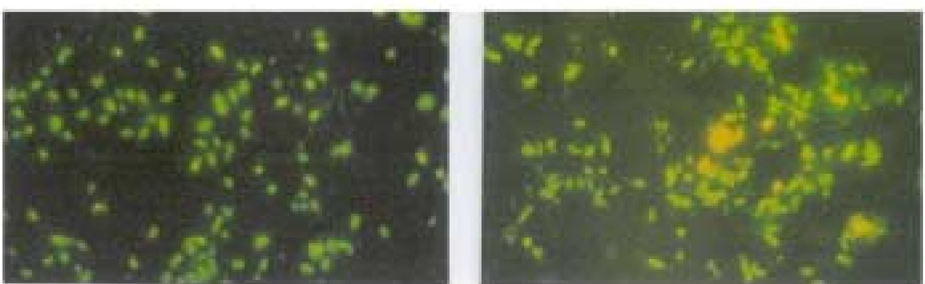
Willcock, L., Allison, D.G., Holah, J.[1], Wirtanen, G.[2] and Gilbert, P.
School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Manchester, UK, [1]Chipping & Chorleywood Food Research Association, Gloucestershire, UK and [2]VTT Biotechnology, Espoo, Finland.

Keywords: Cleansing, Biofilms, Disinfection, Biocides, Fluid dynamic shear

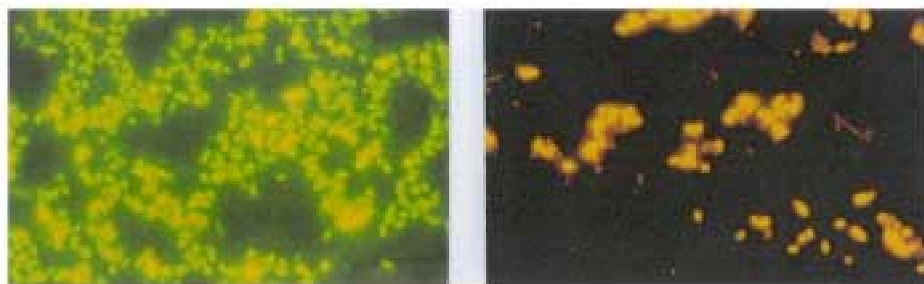
A concentric cylinder reactor (CCR) is described which enables the steady-state kinetics of a microbial biofilms to be evaluated under conditions of constant nutrient flow and variable shearstress. The reactor has been used to evaluate the influence of fluid dynamic shear on the extent and mode of detachment of bacteria from biofilms. Using a food factory isolate of Pseudomonas *aeruginosa* PaENV, a general increase in detachment and overall growth rate of the biofilms ($cfu/cm^2/min^{-1}$) was shown, with time, for each biofilm regardless of the prevailing shear. As the shear rate was increased beyond $0.123ms^{-1}$, then populations tended towards a pseudo steady-state. Sudden changes in shear force, however, caused dramatic changes in the productivity of steady-state populations. The CCR provides an effective means of testing disinfectant activity, particularly for clean-in-place situations and is able to discriminate between biocidal effects and cleansing action for three different chemical classes of disinfectant. Utilisation of the CCR would, therefore, provide enhanced ability to determine the efficacy and efficiency of chemical products for use in sanitation protocols.



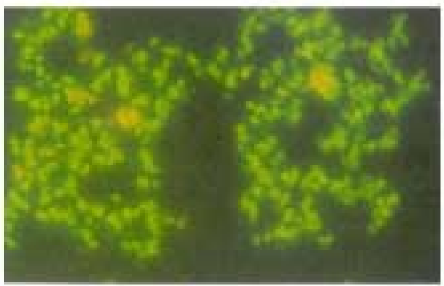
Poster presentation Disinfectant testing using bacteria grown in poloxamer-hydrogel biofilm-constructs based on Bachelor theses by Päivi Härkönen and Mervi Aalto.



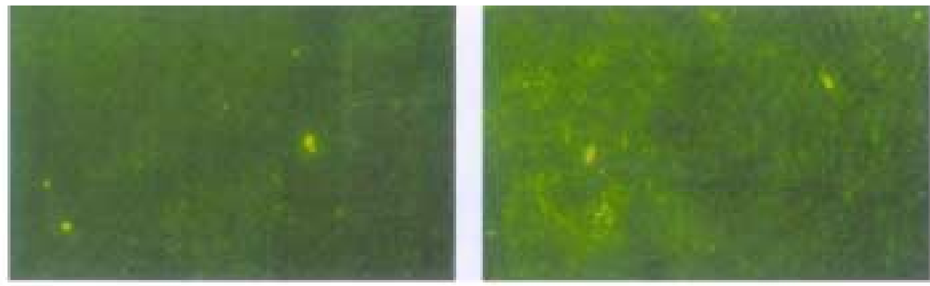
Figures A-B. Spores of Bacillus cereus *229 stained for 2 min with 0.1% erytrosine B (left) and 0.1% acridine yellow (right). The magnification used 1000x.*



Figures C-D. Spores of Bacillus cereus *229 stained for 5 min with 0.1% auramine O (left) and for 15 min with 0.42% trans-4-(p-N,N-dimethylaminostyryl)-N-butoxylcarbonylmethylpyridinium bromide (right). The magnification used 1000x.*



Figures E. Spores of Bacillus cereus *229 stained for 2 min with 0.1% acridine orange. The magnification used 1000x.*



Figures F-G. Spores of Bacillus cereus *229 in milk soil (diluted 1:10) stained for 2 min with 0.1% acridine yellow (left) and for 5 min with 0.1% auramine O (right). The magnification used 1000x.*

Figures H-I. Spores of Bacillus cereus *229 in milk soil (diluted 1:10) stained for 15 min with 0.42% trans-4-(p-N,N-dimethylaminostyryl)-N-butoxylcarbonylmethylpyridinium bromide (left) and for 2 min with 0.1% acridine orange (right). The magnification used 1000x.*

Published by

Vuorimiehentie 5, P.O.Box 2000, FIN–02044 VTT, Finland
Phone internat. +358 9 4561
Fax +358 9 456 4374

Series title, number and report code of publication

VTT Publications 481
VTT–PUBS–481

Author(s)

Wirtanen, Gun, Langsrud, Solveig, Salo, Satu, Olofson, Ulla, Alnås, Harriet, Neuman, Monika, Homleid, Jens Petter & Mattila-Sandholm, Tiina

Title

Evaluation of sanitation procedures for use in dairies

Abstract

The research work for project P96049 in the second NORDFOOD programme was mainly carried out at VTT Biotechnology, Matforsk and SIK together with representatives from the Nordic dairies Valio Ltd., Arla and TINE as well as the technochemical company Suomen Unilever Oy DiverseyLever. The senior advisors at Nordic Industrial Fund involved in the project were Maija Uusisuo and Oddur Gunnarsson. The experiments carried out in the project focused on monitoring methods in sanitation of open and closed systems e.g. fogging, ozonation, footbath hygiene, cleaning of cheese moulds and yoghurt pasteurizers, development of testing procedures for measuring disinfectant efficacy, microbial resistance phenomena against disinfectants, life-cycle assessment and an evaluation procedure for the functionality of the cleaning procedures. New procedures in hygiene have been implemented in dairies based on the results. Development of detection and identification methods for assessing microbial contaminants on or in process equipment, raw material, process air, packaging material and final products is continued in the Nordic dairy hygiene network project DairyNET – Hygiene control in dairy environment, which has partners from all Nordic countries and which is partly funded by the Nordic Industrial Fund (P00027). The contacts between industrial personnel and researchers dealing with hygiene questions in the Nordic countries, which have been built up in the 2 previous NordFood programmes (1994–2000), are thus continued.

Keywords

NORDFOOD, dairies, sanitation, hygiene, microbes, detection, isolation, disinfection, cleaning, life-cycle analysis, environmental assessment

Activity unit

VTT Biotechnology, Tietotie 2, P.O. Box 1500, FIN–02044 VTT, Finland

ISBN	Project number
951–38–6017–5 (soft back ed.) 951–38–6018–3 (URL:http://www.inf.vtt.fi/pdf/)	B1SU00200

Date	Language	Pages	Price
November 2002	English	96 p. + app. 43 p.	C

Name of project	Commissioned by
DairyNET – Hygiene control in dairy environment (P00027)	Nordic Industrial Fund

Series title and ISSN	Sold by
VTT Publications 1235–0621 (soft back ed.) 1455–0849 (URL: http://www.inf.vtt.fi/pdf/)	VTT Information Service P.O.Box 2000, FIN–02044 VTT, Finland Phone internat. +358 9 456 4404 Fax +358 9 456 4374

The research work for project P96049 in the second NORDFOOD programme was mainly carried out at VTT Biotechnology, Matforsk and SIK together with representatives from the Nordic dairies Valio Ltd., Arla and TINE as well as the technochemical company Suomen Unilever Oy DiverseyLever. The senior advisors at Nordic Industrial Fund involved in the project were Maija Uusisuo and Oddur Gunnarsson. The experiments carried out in the project focused on monitoring methods in sanitation of open and closed systems e.g. fogging, ozonation, footbath hygiene, cleaning of cheese moulds and yoghurt pasteurizers, development of testing procedures for measuring disinfectant efficacy, microbial resistance phenomena against disinfectants, life-cycle assessment and an evaluation procedure for the functionality of the cleaning procedures. New procedures in hygiene have been implemented in dairies based on the results. Development of detection and identification methods for assessing microbial contaminants on or in process equipment, raw material, process air, packaging material and final products is continued in the Nordic dairy hygiene network project DairyNET – Hygiene control in dairy environment, which has partners from all Nordic countries and which is partly funded by the Nordic Industrial Fund (P00027). The contacts between industrial personnel and researchers dealing with hygiene questions in the Nordic countries, which have been built up in the 2 previous NordFood programmes (1994–2000), are thus continued.

Tätä julkaisua myy	Denna publikation säljs av	This publication is available from
VTT TIETOPALVELU PL 2000 02044 VTT Puh. (09) 456 4404 Faksi (09) 456 4374	VTT INFORMATIONSTJÄNST PB 2000 02044 VTT Tel. (09) 456 4404 Fax (09) 456 4374	VTT INFORMATION SERVICE P.O.Box 2000 FIN–02044 VTT, Finland Phone internat. +358 9 456 4404 Fax +358 9 456 4374

ISBN 951–38–6017–5 (soft back ed.) ISBN 951–38–6018–3 (URL: http://www.inf.vtt.fi/pdf/)
ISSN 1235–0621 (soft back ed.) ISSN 1455–0849 (URL: http://www.inf.vtt.fi/pdf/)