Suspensions or Biofilms

and other factors that affect disinfectant testing on pathogens

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Proefschrift

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Stellingen

De standaard Europese desinfectietesten kunnen nog op veel fronten verbeterd worden.

*Langsrud, S., and G. Sundheim (1998) Factors influencing a suspension test method for antimicrobial activity of disinfectants. J. Appl. Microbiol. **85**, 1006-1012.*
Dit proefschrift

Bepaling van de membraanintegriteit met behulp van fluorescente stoffen die niet door een intacte celmembraan kunnen dringen, is geen universele vervanger voor de bepaling van bacteriële levensvatbaarheid met behulp van plaattellingen.

*Nebe-von-Caron, G., P. J. Stephens, C. J. Hewitt, J. R. Powell, and R. A. Badley (2000) Analysis of bacterial function by multi-colour fluorescence flow cytometry and single cell sorting. J. Microbiol. Methods. **42**, 97-114.*
Dit proefschrift

De argumenten die Holah *et al.* gebruiken, voor hun bewering dat bacteriën in de praktijk veel minder goed tegen desinfectantia kunnen dan cellen die in een lab gekweekt zijn, kunnen ook gebruikt worden om het tegendeel te beweren.

*Holah, J. T., A. Lavaud, W. Peters, and K. A. Dye (1998) Future techniques for disinfectant efficacy testing. Int. Biodeterior. Biodegrad. **41**, 273-279.*

Slimme en veel geld verdienende mensen zijn langer dan andere mensen.

Volkskrant 13-1-2001, Onderzoek door werknemersorganisatie uit Reykjavik, IJsland Centraal Bureau voor de Statistiek: http://www.cbs.nl/nl/publicaties/artikelen/algemeen/webmagazine/artikelen/1999/0206a.htm

Was mich nicht umbringt, macht mich stärker.

Friedrich Nietzsche

Als je in Wageningen woont, hoef je niet te reizen om iets van de wereld te zien, maar Wageningen schijnt de reislust wel te stimuleren.

Stellingen behorend bij het proefschrift:
Suspensions or Biofilms
and other factors that affect disinfectant testing on pathogens
Suzanne B. I. Luppens
Wageningen, 2002

Contents

Aan mijn ouders en grootouders

Abstract

Disinfectants are very important for the maintenance of proper hygiene in the food industry. In Europe, candidate disinfectants have to be tested on suspended bacteria in so called suspension tests, before they can be approved as disinfectants. In the food industry bacteria usually are attached to surfaces, where they may form biofilms. This mode of growth makes them less susceptible to disinfectants than free-living (suspended) bacteria. Thus, disinfectant testing would greatly profit from a biofilm disinfectant test. The aim of the research described in this thesis was to improve the current European disinfectant tests. To achieve this goal we studied factors that influence the efficacy of disinfectants and alternatives for viability assessment by plate counting. Furthermore, we developed a biofilm disinfectant test.

The bacteria used in this study were the biofilm forming pathogens *Listeria monocytogenes* and *Staphylococcus aureus*. The disinfectants were benzalkonium chloride (BAC), sodium hypochlorite (NaOCl), hydrogen peroxide, and dodecylbenzene sulphonic acid.

The growth phase of cells grown in suspension had a large influence on their susceptibility to disinfectants. *S. aureus* cells grown according to the prescription of the European suspension test were phenotypically not the most disinfectant resistant cells of all the cell types tested. Thus, in suspension tests, cells that are grown differently and have a higher phenotypic resistance than the cells currently used for disinfectant testing could be used.

Fluorescent labeling could be used as a rapid alternative for viability assessment by plate counting, for both free-living cells and biofilm cells, provided the proper fluorescent probes were selected. Thus, for rapid screening of candidate disinfectants, fluorescent probes in combination with flow cytometry may be used instead of plate counting.

In the biofilm disinfectant test, *S. aureus* biofilm formation and biofilm disinfection by BAC and NaOCl were reproducible and a genuine biofilm was produced. To improve disinfectant testing in general, the biofilm disinfectant test developed in this thesis can be added to the set of tests that are used currently. The biofilm test will show which currently used or new disinfectants are the most effective against biofilm cells. In the end, this will contribute to food safety and food quality and the control of cleaning costs in the food industry.

Chapter 1

General introduction

Every year foodborne diseases cause millions of illnesses worldwide [49, 134, 171]. The symptoms include diarrhea, nausea, vomiting and fever. In a limited number of cases there may be severe complications and sequelae such as meningitis, septicemia, arthritis, kidney disfunctioning and even death. It is therefore necessary to guard the microbiological quality of our food very strictly. Bacteria are always present on and in raw foods. Historically, numerous processing techniques have been developed to kill, inactivate or prevent further growth of pathogenic and spoilage bacteria. These techniques include cooking, pasteurization, salting and fermentation. New techniques such as pulsed electric field (PEF), high pressure treatment and modified atmosphere packaging are currently being developed and introduced [5, 6, 98, 151]. However, when the bacteria from the raw food have been killed or inactivated food can still be recontaminated with bacteria. Thus, everything that comes into contact with food should be free of bacteria or have a level of contamination that is not harmful. This applies to all food contact surfaces and processing equipment in the food industry. Therefore, these surfaces have to be cleaned and disinfected on a regular basis to prevent accumulation of pathogenic and spoilage organisms. Significant problems are posed by bacteria that can form biofilms on these surfaces, since biofilm cells are difficult to remove and are more resistant to disinfection than free-living bacteria [50, 181]. Disinfectants are traditionally tested on cells in suspension (free-floating or planktonic cells) [9, 10]. Thus, disinfectant testing would greatly profit from the inclusion of a biofilm disinfectant test.

The literature survey below gives an introduction to biofilms, the human pathogenic bacteria used in this thesis: *Staphylococcus aureus* and *Listeria monocytogenes* which are both known biofilm formers, an overview of disinfectants and how they are tested and an overview of fluorescent techniques that can be used as an alternative for the classical plating assays used for viability testing after exposure to disinfectants.

BIOFILMS

A biofilm is a slime layer with microorganisms, or to be more exact a community of microorganisms embedded in an organic polymer matrix, adhering to a surface [41]. Examples of places were biofilms can be found are as diverse as teeth, ship hulls, prosthetic implants, (drinking) water systems (see Fig. 1.1) and food contact surfaces [20, 41, 91, 112, 129, 189]. In short, biofilms can be found in all places were the following requirements are met: presence of a surface, nutrients, moisture, a suitable temperature and microorganisms. Temperature amongst others influences which microorganisms will form a biofilm, as was

Figure 1.1 Example of biofilm formation in an industrial water system.

shown by Langeveld *et al.* [113]. Above mentioned examples are all places were biofilm formation is detrimental. In those places biofilms cause energy losses and deterioration or create health risks [112, 129, 189]. However, biofilms can also be useful, for instance in the human gut and when they are used for wastewater treatment, bioremediation or the production of vinegar and ethanol [91, 112].

The formation of a biofilm can be divided into several steps: Approach of planktonic cells to the (preconditioned) surface, reversible attachment, irreversible attachment, microcolony formation, biofilm formation with production of extracellular polymeric substances and finally detachment of cells [112]. This sequence of events is mostly based on research of biofilms formed by Gram-negative bacteria, the most studied biofilm formers. Fig. 1.2. is a diagrammatic representation of a biofilm based on studies with Gram-negatives.

It is generally recognized that cells in biofilms are more resistant to disinfectants compared to their planktonic counterparts [37, 112]. There are several causes for the increased resistance of biofilm cells: (i) the extracellular material of the biofilm excludes or influences the access of the disinfectant, (ii) the outer layers of the biofilm may react with and quench the disinfectant, (iii) attachment changes the physiology of the bacteria, (iv) limited availability of key nutrients within the biofilm results in alteration of the physiology of the bacteria and induction of stress response [37, 112], (v) the high cell density in the biofilm results in expression of different phenotypes [56]. All of these parameters have been shown to be applicable to biofilms, but not all for the same biofilm or for a specific antimicrobial [15, 37, 56, 62, 84]. It has been suggested that the structure of the biofilm can moderate the delivery of an antimicrobial agent to cells within the biofilm which may give the cells time to adopt a physiologically protective set of changes against the antimicrobial agent [112, 168]. In recent years research has focussed on the last three of the five above-mentioned parameters.

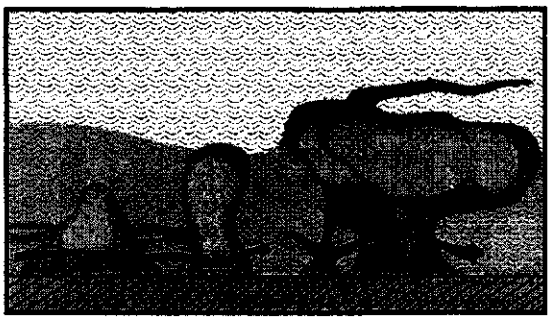

Figure 1.2 Diagrammatic representation of a microbial biofilm showing the organization of this adherent population, in terms of microcolonies and water channels, and the convective flow within these channels. Taken from Costerton *et al.* [50].

To study biofilms, well-defined setup- and biofilm formation-protocols are needed. Several methods have been described for growing laboratory biofilms. A simple method that is suggested to yield biofilms is to spread a cell suspension on coupons and let them dry [29]. However, this approach only allows attachment of cells to a surface, but does not allow them to grow on the surface to form a real biofilm. Another method is to place coupons in inoculated medium and replace the medium several times, or to use inoculated medium for development of biofilms on the surface of microtiter plate wells or Erlenmeyer flasks (batch systems) [24, 46]. A disadvantage of these methods is that the cells will be very loosely attached to the surface. An interesting method is to trap planktonic cells in a poloxamer-hydrogel that is liquid at temperatures below 15 °C and solid at temperatures above 15 °C [82]. The advantage of this method is that viability of cells can be easily analyzed. The disadvantage is that the cells do not have the biofilm physiology. Another method is to apply cells on a filter and place this on solid medium [180] or perfuse the filter with liquid medium [76]. These biofilms are different from natural biofilms on inert surfaces because the cells receive their nutrients from the side of the surface and not from the side of the air or bulk liquid like normal. The methods that come closest to biofilms in practice are the ones that are formed in special reactors that apply a certain shear force on the biofilm cells while they are growing (continuous systems). Examples of this kind of reactors are the Robbins device [2], a chemostat with coupons in it [191], the constant depth film fermenter [177] and the culture container that is used in Chapter 5 of this thesis [124].

Biofilm formation can be detected in several ways. The presence of a biofilm can be indirectly monitored with a Malthus microbiological growth analyzer (based on changes in conductance) [71] or by detection of cell metabolites or cell constituents such as ATP, proteins or exopolysaccharides [81, 88, 186]. Direct monitoring can be done by removal of biofilm cells from the surface by swabbing, vortexing with glass beads, scraping or by sonication and further analysis by plating or possibly flow cytometry [41, 88, 124]. Intact biofilms can be analyzed with phase contrast microscopy, electron microscopy, confocal laser

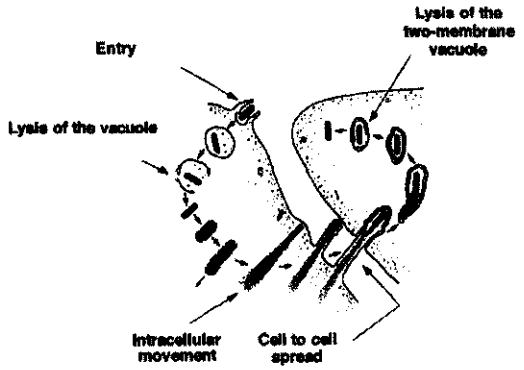

Figure 1.3 Schematic representation of cell invasion by *L. monocytogenes*.

scanning microscopy, fluorescence microscopy (autofluorescent cells, green fluorescent protein or fluorescently labeled cells), direct viable count or radio-active labeling of cells [50, 87, 88, 89, 94, 132, 167, 186, 190].

LISTERIA MONOCYTOGENES

L. monocytogenes is a Gram-positive non-sporeforming facultatively anaerobic rod, motile by means of flagella. *Listeria* was named after Lord Lister, an English surgeon. *Monocytogenes* means monocyte (blood cell) producing. This refers to the ability of *L. monocytogenes* to infect blood cells (see Fig. 1.3) [160]. Some studies suggest that 1-10 % of humans may be intestinal carriers of *L. monocytogenes*. The bacterium has been found in mammals, birds and in some species of fish and shellfish. It can be isolated from soil, silage and other environmental sources and in food processing plants [117, 143, 144]. *L. monocytogenes* is quite hardy and resists the deleterious effects of freezing, drying and heat remarkably well for a bacterium that does not form spores. *L. monocytogenes* has been associated with such foods as raw milk, supposedly pasteurized milk, cheeses, ice cream, raw vegetables, fermented raw-meat sausages, raw and cooked poultry, raw meats and raw and smoked fish. Its ability to grow at temperatures as low as 0 °C permits multiplication in refrigerated foods [12, 68, 69, 97, 160].

L. monocytogenes causes listeriosis, which manifests itself in septicemia, meningitis, encephalitis and intrauterine or cervical infections in pregnant women, which may result in spontaneous abortion or stillbirth. The onset of these disorders is usually preceded by influenza-like symptoms including persistent fever. Gastrointestinal symptoms may precede the more serious forms or may be the only symptoms expressed. Overall mortality for listeriosis may be as high as 23 % [162]. The main target populations for listeriosis are pregnant women, immunocompromised people, the elderly and sometimes healthy

individuals, particularly when food is heavily contaminated with the organism. The infective dose of *L. monocytogenes* is believed to vary with the strain and susceptibility of the victim. From cases contracted through milk it has been concluded that in susceptible persons fewer than 1,000 total organisms may cause disease [12, 68, 69, 97, 160].

L. monocytogenes can form biofilms on surfaces of materials that are present in the food industry, such as stainless steel, glass, cast iron and plastic [27, 74, 101, 111, 121, 130, 143, 159, 166]. Its biofilm formation has been mostly studied in batch systems [27, 74, 101, 111, 121, 130, 143, 159, 166]. Inhibition or enhancement of *L. monocytogenes* growth in mixed species biofilms has also been studied [117, 118, 144]. *L. monocytogenes* biofilm cells are more resistant to quats, acid anionics, hypochlorite, iodine, peroxyacetic acid and phenol [75, 121]. Despite the multitude of studies on *L. monocytogenes* biofilms, only few have focused on the genetic basis of attachment and biofilm formation by this foodborne pathogen. With the help of a flagellin mutant it was shown that flagella facilitate the early stage of attachment of *L. monocytogenes* to stainless steel [173].

STAPHYLOCOCCUS AUREUS

S. aureus is a Gram-positive, non-sporeforming facultatively anaerobic spherical bacterium. The word 'staphylococcus' is derived from 'staphyle' which is Greek for bunch of grapes and 'coccus' which means grain or berry. The name refers to the grape bunch-like clusters that are formed by *S. aureus* during growth and the spherical form of the bacterium (see Fig. 1.4). 'Aureus' means golden which refers to the yellow color of *S. aureus* colonies on plates [108].

Staphylococcal food poisoning or staphyloenterotoxicosis is caused by the heat stable enterotoxins that some *S. aureus* strains produce under favorable growth conditions [97]. The most common symptoms are nausea, vomiting, retching, abdominal cramping and prostration. In more severe cases, headache, muscle cramping and transient changes in blood pressure and pulse rate may occur. A toxin dose of less than 1.0 microgram in contaminated food will produce symptoms of staphylococcal intoxication. This toxin level is reached when *S. aureus* populations exceed 100,000 per gram. *S. aureus* is also involved in toxic shock syndrome and is a common cause of community-acquired infections including endocarditis, osteomylitis, septic arthritis, pneumonia and abscesses [13, 97].

Foods that are frequently incriminated in staphylococcal food poisoning include meat products, poultry and egg products, salads, bakery products, sandwich fillings and milk and dairy products. Foods that require considerable handling during preparation and that are kept at slightly elevated temperatures after preparation are frequently involved in staphylococcal food poisoning [13, 97].

Staphylococci exist in air, dust, sewage, water, milk and food or on food equipment, environmental surfaces, humans and animals. Humans and animals are the primary reservoirs. Staphylococci are present in the nasal passages and throats and on the hair and

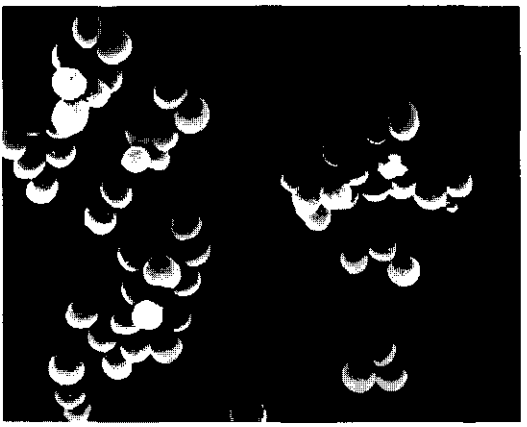

Figure 1.4 Scanning electron microscopy image of *S. aureus*. Taken from Zoltai *et al.* [192].

skin of 50 percent or more of healthy individuals. Although food handlers are usually the main source of food contamination in food poisoning outbreaks, equipment and environmental surfaces can also be sources of contamination with *S. aureus* [13, 24, 97].

Most studies on biofilm formation by *S. aureus* have been done with surfaces that are used in the medical field. This is because *S. aureus* is one of the main bacteria that forms biofilms on prosthetic devices and in this way causes infections that are very difficult to treat with antibiotics [24]. *S. aureus* forms biofilms on a variety of surfaces: hydroxyapatite, stainless steel, glass and on all kinds of plastics like polystyrene, polypropylene and silicone [14, 52, 81, 99, 124, 146, 152]. On most of these materials biofilms have been grown statically in a batch system, with sometimes one to three medium refreshments [3, 53, 81, 146, 185]. Especially in genetic studies batch biofilm formation in microtiter plate wells is very popular [14, 53, 73, 154]. In the batch systems biofilm formation varied from 5×10^6 to 8×10^7 CFU cm^{-2} after 24 to 48 h. *S. aureus* biofilms grown in chemostat varied from 3×10^6 CFU cm^{-2} after 4 days to 2×10^8 CFU cm^{-2} after 13 days [14]. *S. aureus* biofilm cells are more resistant to antibiotics [4, 14, 185], disinfectants [146] and enzymes [99]. It is speculated that the resistance to enzymes may depend on the thickness of the biofilm [99] and resistance to antibiotics may be due to alteration of the physiological status of the cells such as altered membrane permeability for antibiotics, alteration of molecular targets of antibiotics, induction of antibiotic degrading enzymes, exopolysaccharide production and slow growth [14, 185].

Recently, some studies have focussed on the genes that are involved in *S. aureus* biofilm formation on abiotic surfaces. *S. aureus* biofilm formation consists of two steps: adhesion of bacteria to a surface followed by cell-cell adhesion, which results in a multiple layer biofilm [52]. Disruption of the *arlR/arlS* locus which codes for a two-component regulatory system or disruption of *sar* (staphylococcal accessory regulator) results in increased adherence of *S. aureus* (first step) [73, 152]. Bap (biofilm associated protein), a *S.*

aureus surface protein, is involved in both steps [53]. The *ica* locus that encodes for the polysaccharide intercellular adhesin (PIA) which is composed of linear β-1,6-linked glucosaminylglycans is involved in the second step [52]. Furthermore, several studies show that stress is involved in *S. aureus* biofilm formation. Osmotic stress induces biofilm formation and *ica* transcription [154] and anaerobic growth stimulates PIA production [51]. Additionally, Becker *et al.* [24] showed that homologues to genes encoding glycolytic enzymes and other metabolic enzymes (phospoglycerate mutase, triosephosphate isomerase and alcohol dehydrogenase) are upregulated in biofilms; this may be correlated to oxygen limitation under these conditions. Of two other upregulated genes in *S. aureus* biofilms, one showed homology to ClpC, a general stress protein, and the other to threonyl-tRNA synthetase that is indicative of threonine starvation [24].

DISINFECTANTS

To prevent accumulation of pathogenic and spoilage organisms food contact surfaces have to be cleaned and disinfected on a regular basis. For that purpose cleaning agents and disinfectants are being used. A cleaning agent is used to facilitate the removal of adhering organic material and soil. In dictionary definitions of what a disinfectant is there are five recurring elements: it is an agent that removes infectious agents, kills micro-organisms, may not kill spores, may be chemical or physical and is used on inanimate objects [28]. The British Standard Institution defines disinfection as the destruction of vegetative microbial cells; bacterial spores are generally not affected. A disinfectant does not necessarily kill all microorganisms, but reduces their numbers to a level acceptable for a defined purpose; for example, a level which is neither harmful to health nor to the quality of perishable foods [29].

Disinfectant efficiency is increased when it is applied to a surface that first has been cleaned. A wide range of disinfectants is used in the food industry. These disinfectants can be divided in different groups: oxidizing agents such as chlorine-based compounds, hydrogen peroxide, ozone and peracetic acid; surface active compounds such as quaternary ammonium compounds and acid anionics; iodophores and enzymes [186]. The efficiency of disinfection is influenced by interfering organic substances, pH, temperature, concentration and contact time [186]. Desired characteristics of disinfectants are that they must be effective, safe, easy to use and easily rinsed off surfaces leaving no toxic residues or residues that affect the sensory properties of the product [186].

In the research described in this thesis four disinfectants were used: hypochlorite, hydrogen peroxide, benzalkonium chloride and dodecylbenzene sulphonic acid. Hypochlorite is a chlorine-based oxidizing agent. Its mechanism of action is reaction with and alteration of proteins and DNA, especially enzymes with thiol or amino groups [64, 66, 158]. Its recommended concentrations in the food industry range from 2 mg l^{-1} for rinse and cooling water to 5,000 mg l^{-1} for concrete surfaces. Hypochlorite is cheap, effective, easy to use, it

detaches the biofilm matrix and it has a broad spectrum of activity. Disadvantages are its poor stability, its toxicity, corrosiveness and its lack of initial adhesion control, discoloration of the product and occurrence of rapid aftergrowth [64, 186].

Hydrogen peroxide is an oxidizing agent. Its mechanism of action is oxidation or formation of free radicals [1, 86] which affect enzymes and proteins, DNA, membranes and lipids resulting in damage of transport systems and receptors, difficulty in maintaining ionic gradients over the cytoplasmic membrane, impairment of replication and (in)activation of enzyme systems [1, 86, 158]. Hydrogen peroxide decomposes to water and oxygen, is relatively non-toxic, can easily be used *in situ*, weakens the biofilm and supports biofilm detachment. Disadvantages are that high concentrations are necessary and it is corrosive [186].

Benzalkonium chloride (BAC) is a quaternary ammonium compound (a quat). Its mechanism of action involves alteration of the semi-permeable properties of cell membranes, which leads to leakage of metabolites and coenzymes and disturbance in the delicate balance of metabolite concentrations within the cell [135]. The recommended concentrations of BAC in the food industry range from 200 to 800 mg l^{-1} depending on the type of surface [186]. The advantages of quats are that they are effective at non-toxic concentrations, support biofilm detachment and prevent growth, are non-corrosive, non-irritating and have no flavor or odor. The disadvantages are that they are inactivated by low pH, calcium salts and magnesium salts, are relatively ineffective against Gram-negatives and microorganisms may develop resistance against them [129, 186].

Dodecylbenzyl sulphonic acid (DSA) is an acid anionic surfactant. To DSA solutions phosphoric acid is added to increase its performance [65] because at neutral pH DSA is negatively charged and thus repelled by the negatively charged bacterial surface [65]. The mechanism of action at low pH is disorganization of the cell membrane, inhibition of key enzyme activities and interruption of cellular transport and denaturation of cellular proteins [65, 158]. Advantages of DSA use are it has cleaning/detergent properties, it removes particles from surfaces, it has a broad spectrum of activity, it is noncorrosive and does not stain equipment, gives no odor, it is stable, works rapidly and has residual bacteriostatic activity. Disadvantages are that it is only effective at low pH, it generates foam and it has a slow activity against spores [65, 186].

DISINFECTANT TESTING

The procedure for the testing of candidate disinfectants in Europe consists of 3 phases. In phase 1 the basic activity of the product is tested in a suspension test. Phase 2 consists of two steps. In the first step the product is tested in a suspension test under conditions representative of different practical uses. The second step consists of other laboratory tests e.g. handwash,

handrub and surface tests simulating practical conditions. Phase 3 consists of field tests under practical conditions [10].

Up till now all European tests for disinfectants used in the food, industrial, domestic and institutional areas are suspension tests. In these bacterial suspension tests a suspension is made from colonies grown on solid medium. The main organisms that are used for these tests are *Pseudomonas aeruginosa* ATCC 15442, representative for the Gram-negative bacteria and *Staphylococcus aureus* ATCC 6538 representative for the Gram-positive bacteria. The suspended bacteria are exposed for 5 (phase 2) or 60 min (phase 1) to the candidate disinfectant [9, 10]. Then the disinfectant is neutralized and bacterial survival is determined by plate counting. If the candidate disinfectant reduces the concentration of viable cells by more than 5 log units and the concentration of cells in the suspension is within a certain limit, it is approved as a disinfectant.

However, a good disinfectant test must be able to predict the value of the disinfectant in practice [156] and in practice cells can be found much more frequently on surfaces than in suspension. Thus, it is questionable whether suspension test cells are really representative of the cells found in practice. In this light a new surface test is being developed for phase 2 step 2 [29, 107]. In this surface test a suspension of cells is put on a surface and dried for one hour. Then the disinfectant is applied. This surface tests is already a step forward compared to the suspension tests. Still there can be some concern about the suitability of this surface test. The cells in a surface test only attach to the surface and do not grow, whereas it is known that attached cells that are allowed time to grow form biofilms.

Therefore, a standard biofilm test would be a very useful addition to the current tests. Such a standard test has to meet several requirements. In the first place a disinfectant test should be as simple as possible and not require specialized or expensive pieces of laboratory equipment. Furthermore, the test should be repeatable and reproducible [90]. Several studies use laboratory biofilm systems that have been designed to evaluate biocides in specific environments, for instance the human mouth, toilet bowls, oilfield water injection systems and cooling water or water distribution systems [63, 150]. Recently, a few studies have focused on a general test for disinfectant testing or, more general, biocide susceptibility testing. Gilbert *et al.* [79] use a batch system, a 96 well microtiter plate, for their test. They do not describe the reproducibility of biofilm formation in this system. Ceri *et al.* [42] use a shaking batch system, the MBEC assay system that consists of a 96 well microtiter plate with in each well a peg (on which the biofilm is formed) attached to a second plate. The reproducibility of biofilm formation in this system is good, but the reproducibility of disinfection is not mentioned. Willcock *et al.* [183] use a concentric cylinder reactor with variable nutrient flow and variable shear stress to evaluate cleaning in place strategies. They do not describe the reproducibility of biofilm formation in this system. Zelver *et al.* [191] use a rotating disk reactor, which is a chemostat with a rotating disk that contains removable disks at the bottom. They show that disinfection of their biofilms with hypochlorite gives reproducible results. However, their reactor contains only six disks, which is a very low

number, especially if several disinfectants have to be tested on the same batch of biofilms. All of the systems described above, except perhaps the rotating disk reactor and the concentric cylinder reactor, are relatively simple and do not require very specialized or expensive pieces of laboratory equipment.

FLUORESCENCE TECHNIQUES

The traditional method to determine viability of bacteria, also in disinfectant testing, is plate counting. This method is based on the reproductive capacity of cells. However, this method has some disadvantages. The plate count technique requires long incubation times (2 days). Furthermore, for viability assessment of attached or biofilm cells, the cells have to be removed from the surface for analysis. Additionally, several studies report that cells are metabolically active and potentially able to cause disease [30] while they are incapable of the cellular division required to form a colony on a plate. This is also known as a viable but non culturable (VBNC) state [32, 48, 102, 120] or better: an active but non culturable state (ANC) [100]. In the case of disinfectants this may lead to the overestimation of the efficacy of the disinfectant.

An alternative method to determine viability is labeling with fluorescent probes. These probes indicate if a cell possesses other physiological characteristics required for a cell to be viable, such as membrane integrity, enzyme activity and energy production [34, 100]. Fluorescent probes can be used directly to assess viability of attached cells when their use is combined with fluorescence microscopy (thin layers) [40, 130, 131, 190] or confocal scanning laser microscopy (thick layers) [50, 83, 133, 177]. Fluorescent labeling can also be combined with flow cytometry (FCM). In this way viability assessment can be done in 0.5 to 2 hours. FCM is a technique for individual cell analysis and it has been applied earlier to analyze heterogeneous populations such as starved cells [179] and attached cells [184] and may be used for biofilm cell analysis. Fluorescent labeling is influenced by the growth phase of bacteria [31, 32, 48, 96, 163, 169, 184]. As cells in biofilms face nutrient gradients [26, 57, 188] and are therefore in different growth phases or have differing growth rates [167, 188] it is recommended to first test the suitability of fluorescent probes for viability assessment of free-living cells in different growth phases after exposure to disinfectants.

Several categories of fluorescent probes are used for detection and viability assessment of micro-organisms [22, 33, 34, 100, 141]. **Membrane potential probes** are compounds such as rhodamine 123, oxonol and carboxycyanine dyes. Some of these probes can be extruded actively out of the cell and loss of membrane potential does not necessarily indicate cell death [34, 140]. **pH probes**, for instance (carboxy)fluorescein, carboxyfluorescein succinimidyl ester (CFSE), BCECF, calcein, SNARF-1 and pyranine are used to determine the internal pH of micro-organisms. Maintenance of a pH gradient (i.e. pH_{in} higher than pH_{out}) is indicative of cellular activity and membrane integrity.

Disadvantages of some of these probes are that they are extruded actively out of the cell [33, 35, 123]. **Redox indicators** are CTC, INT and Alamar Blue. Results with these probes depend on endogenous and exogenous substrates, may be independent of the electron transport system and the probes may be toxic at higher concentrations [47, 161, 163, 164]. **Enzyme substrates** such as (carboxy)fluorescein diacetate, fluorescein digalactoside and Chemchrome B are used to indicate enzyme activity. These non-fluorescent substrates are converted to fluorescent products and then they can be detected. These products are often used as **dye retention probes**. Dye retention is an indication for an intact cell membrane. Disadvantages of these probes are that the dyes can be actively extruded, enzyme activity could be absent or poor under certain physiological conditions, enzyme activity is typically energy-independent and the dye can sometimes by retained in vacuoles, even when the membrane is permeable [22, 100, 141, 172]. **Dye exclusion probes** are for example propidium iodide, ethidium homodimer, SYTOX Green, TOTO and TO-PRO-3. These probes are only able to penetrate cells with compromised membranes. Unfortunately, some of them are able to label intact cells under certain conditions [141]. Furthermore, some live cells have permeant membranes and some dead cells have impermeant membranes [123, 141]. **Nucleic acid stains** such as Hoechst 33258/33342, DAPI, SYTO series and YOYO are able to penetrate the cell membrane of all cells. Other nucleic acid stains, such as acridine orange, first require fixation of the cells [180]. Nucleic acid stains are used to detect the total number of cells or to quantify the amount of nucleic acid. Of course with these probes one must take into account that the amount of target nucleic acid depends on the physiological state of the cell [22]. **Fluorescent *in situ* hybridization (FISH) probes** are for instance 16S rRNA-directed probes. These probes are used to assess ribosomal content of microorganisms and to detect different species in environmental samples [22, 100].

OUTLINE

The aim of the work described in this thesis is to study the effects of disinfectants on cell constituents and viability of pathogenic bacteria and the factors that influence the efficacy of disinfection, and with this knowledge to improve the current disinfectant tests.

In **Chapter 2** the effect of BAC on viability and cell constituents in *L. monocytogenes* was studied. Furthermore the influence of several factors on disinfectant efficacy was studied. In **Chapter 3** the effect of the growth phase of *S. aureus* on the efficacy of four disinfectants was studied. The susceptibility of the cells in different growth phase was compared to that of the suspension test cells that are currently used for disinfectant testing. In **Chapter 4** fluorescent probes in combination with flow cytometry were used for rapid study of the effect of disinfectants on single *L. monocytogenes* cells. The viability of the cells determined by fluorescent techniques was compared to viability determined by plate counting. In **Chapter 5** a standard biofilm test was developed for *S. aureus* and the resistance of the biofilm test cells

was compared to that of the currently used suspension test cells. **Chapter 6** discusses viability assessment after exposure to disinfectants and the factors that influence the efficacy of disinfection that were found in this thesis. In addition, it gives recommendations for improvement of disinfectant tests, it integrates the results from this thesis with results from other studies and gives some perspectives for biofilm control in practice.

Chapter 2

Effect of benzalkonium chloride on viability and energy metabolism in exponential- and stationary-growth phase cells of *Listeria monocytogenes*

S.B.I. Luppens, T. Abee, J. Oosterom

The difference in killing exponential- and stationary-phase cells of *Listeria monocytogenes* by benzalkonium chloride (BAC) was investigated by plate counting and linked to relevant bio-energetic parameters. At a low concentration of BAC (8 mg l^{-1}) a similar reduction in viable cell numbers was observed for stationary-phase cells and exponential-phase cells (an approximately 0.22 log unit reduction), although their membrane potential and pH gradient were dissipated. However, at higher concentrations of BAC, exponential-phase cells were more susceptible than stationary-phase cells. At 25 mg l^{-1} the difference in survival on plates was more than 3 log units. For both types of cells killing, i.e. more than 1-log unit reduction in survival on plates, coincided with complete inhibition of acidification and respiration and total depletion of ATP pools. Killing efficiency was not influenced by the presence of glucose, brain heart infusion medium, or oxygen. Our results suggest that growth phase is one of the major factors that determine the susceptibility of *L. monocytogenes* to BAC.

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Chapter 2

INTRODUCTION

Listeria monocytogenes is a ubiquitous Gram-positive foodborne pathogen that can cause life-threatening illness in immunocompromised and elderly people, pregnant women, and neonates [68, 69, 87]. It has been isolated from different sources in the environment and foods [68, 69]. One of its characteristics is that it can grow at very low temperatures, down to 0 °C [21]. *L. monocytogenes* is able to attach to and form extracellular material on the types of surfaces present in food industry and in households [27, 74, 85, 105, 111, 126, 127, 157]. The attached cells are more resistant to disinfectants than free living cells [74, 139, 157]. Consequently, these cells are more difficult to eradicate, and the hazard of food contamination increases. Therefore, it is necessary to know more about biofilm-forming bacteria, their survival mechanisms, their resistance to disinfectants, and other factors that influence the effectiveness of disinfection.

There are several ways to explain the increased resistance of biofilm cells: (i) the extracellular material of the biofilm excludes or influences the access of the disinfectant, (ii) the outer layers of the biofilm may react with and quench the disinfectant, (iii) attachment changes the physiology of the bacteria, (iv) limited availability of key nutrients within the biofilm alters the physiology of the bacteria [37]. The fourth resistance theory presumes the formation of oxygen gradients and gradients of other nutrients in a biofilm. The existence of these gradients has been confirmed in several studies [26, 57, 188]. Because of these gradients, cells at the inside of the biofilm lack a sufficient amount of essential nutrients. Free-living cells are known to respond to this nutrient stress by growth rate reduction and induction of defense mechanisms (stress response) [72, 93, 110]. As a result, they become more resistant to other types of stress [109, 128, 155]. The fourth resistance theory supposes that the same is true for biofilm cells. Recently, several studies have confirmed that in biofilms cell proliferation is reduced and stress responses are induced [2, 136, 167, 188]. For example the alternative σ-factor for RNA polymerase, rpoS, plays an important role in the biofilm formation of *Escherichia coli* and *Salmonella enteritidis* [2, 136]. Apparently, there exist considerable similarities between responses of biofilm cells and free-living cells, in particular stationary-phase cells that suffer from a lack of nutrients [55].

In this study, we investigated the effect of benzalkonium chloride (BAC), a quaternary ammonium compound (quat), on exponential-phase cells and stationary-phase cells of *L. monocytogenes*. Quats are membrane active compounds [59, 135] used as disinfectants in the food industry [186]. We compared the resistance of stationary-phase cells with that of fast growing exponential-phase cells to investigate whether there were large differences in disinfectant susceptibility between nutrient stressed cells and unstressed cells. We did this by investigating the dose-response relation of *L. monocytogenes* exponential-phase and stationary-phase cells to BAC. Furthermore, we investigated the influence of brain heart infusion medium (BHI), glucose, and oxygen on killing efficiency, and we discuss

whether differences in growth phase may contribute to the increased resistance of biofilm cells to quats.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *L. monocytogenes* Scott A was grown at 30 °C in BHI broth with 0.5 % wt/vol glucose. Stock cultures were kept at -80 °C with 25 % wt/vol glycerol added. An overnight culture of *L. monocytogenes* that was statically grown was used to inoculate 30 ml of fresh medium (2 % vol/vol inoculum). This new culture was shaken in a gyrorotatory incubator at 150 rpm in a 100 ml Erlenmeyer flask. Exponential-phase cells were harvested at an $O.D._{620\ nm}$ of 0.20 (after approximately 3.5 h). Stationary-phase cells were harvested after 15.5 h of growth. Before use, cells were washed twice by centrifuging for 10 min at 2620 × *g* and resuspending in phosphate buffered saline (PBS) (0.2 g of KCl, 0.2 g of KH_2PO_4, 1.5 g of Na_2HPO_4 and 8.0 g of NaCl per liter, adjusted to pH 7.2 with HCl). Cells were resuspended to a concentration ranging from 5×10^8 to 1×10^9 CFU ml^{-1} in the buffer that was used for the particular experiment (see below), with the exception of cells used for the measurement of acidification and respiration, which were concentrated 90 times and 40 times more, respectively. All experiments and assays were performed at 30 °C.

Chemicals and disinfectant. BHI was obtained from Difco Laboratories (Detroit, USA). Fluorescent probes were obtained from Molecular Probes Europe B.V. (Leiden, The Netherlands). Glycerol was from Fluka Chemie AG (Buchs, Switzerland), resazurin from Janssen (Geel, Belgium) and nigericin, valinomycin and bovine serum albumin from Sigma Chemical Co. (St. Louis, USA). All other chemicals were from Merck KGaA (Darmstadt, Germany). The disinfectant used in this study, BAC (Alkyl-benzyl-dimethylammonium chloride, alkyl distribution from C_8H_{17} to $C_{16}H_{33}$), is a quat and a cationic surfactant. It was purchased from Lamers & Pleuger B.V. (Den Bosch, The Netherlands). A 50 g l^{-1} stock solution of BAC was made in demineralized water and filter sterilized through a 0.2 µm filter (FP 030/03 Schleicher and Schuell GmbH, Dassel Germany). A 10 × dilution in demineralized water was prepared from this stock solution before each experiment and used immediately.

Killing experiments. Killing experiments were performed in 5 ml PBS. For the determination of the influence of glucose and BHI medium on killing PBS, PBS with 0.5 % wt/vol glucose (PBS-g) and BHI with in total 0.5 % wt/vol glucose (BHI-g) were used. A time zero sample was taken to determine the initial number of cells. Then 5 g l^{-1} BAC solution was added to produce final concentrations ranging from 8 to 25 mg l^{-1} and the cell suspension was mixed. Five min after addition a sample was taken and immediately diluted 10 times in PBS. An appropriate dilution of the sample was made immediately in PBS and cells were enumerated by spiral-plating on BHI-agar with 0.5 % wt/vol glucose immediately after dilution. The plates were incubated at 30 °C, and the colonies were counted after two

days of incubation. A similar protocol as described above was used for the killing dependent on time. Additional samples were taken after 10, 15, 20, 25 and 30 min. Killing by BAC in the absence and presence of oxygen was performed as described above, with the following modifications. Experiments were performed with PBS and BHI agar containing cysteine (0.5 g l^{-1}) and resazurin (1 mg l^{-1}) to demonstrate lack of oxygen. Bacteria were kept on ice before use. Killing in the absence of oxygen was performed in an anaerobic glove box (containing 80 % nitrogen gas, 10 % hydrogen gas, and 10 % carbon dioxide). Bacterial cells exposed to BAC in the absence of oxygen were incubated at 30 °C in an anaerobic jar, and colonies were counted after 3 days, because of their slower growth. Killing experiments dependent on concentration were done in duplicate on different days. Killing experiments dependent on time were done in triplicate on different days. Killing experiments to establish the influence of glucose, BHI medium or oxygen were done in triplicate on the same day, and paired Students *t* test with two tailed distribution and a 0.05 confidence level was used to analyze all data. The null hypothesis was that there was no significant difference between the killing in absence or presence of oxygen, glucose or BHI medium. For all experiments, the average value for survival is given in the graphs.

Measurement of membrane potential and intracellular pH. Membrane potential was measured using the fluorescent probe $DiSC_3[5]$ (3,3-dipropylthiadicarbocyanine; excitation wavelength, 643 nm; emission wavelength, 666 nm). The distribution of this probe is membrane potential dependent; the higher the membrane potential is, the more the probe accumulates in the cell. Fluorescence is quenched when the probe is inside the cell. Assays were performed with 8 µmol l^{-1} $DiSC_3[5]$ in 3 ml of a 50 mmol l^{-1} potassium phosphate buffer, pH 7.2 (KPi). This is a qualitative measurement.

The intracellular pH was measured using the fluorescent probe CFDA,SE (5(and 6-) carboxyfluorescein diacetate succinimidyl ester) as described by Breeuwer *et al.* [35]. Washed cells were incubated for 10 min with 0.3 µmol l^{-1} CFDA,SE and washed in KPi. Glucose (final concentration 0.003 % wt/vol) was added to the cells to eliminate non-conjugated CFSE (5(and 6-) carboxyfluorescein succinimidyl ester), and the cells were incubated for 30 min. Cells were washed and resuspended in KPi. Assays were performed in 3 ml of the same buffer. Cells were kept on ice prior to use. Fluorescence intensities were measured at excitation wavelengths of 500 and 440 nm and the emission wavelength was 530 nm. The internal pH was calculated from the 500-to-440 nm ratio with the use of a calibration curve. For both pH gradient and membrane potential measurements, fluorescence was measured in the stirred and thermostated cuvette holder of a Luminescence spectrometer LS 50B (Perkin-Elmer, Nieuwerkerk aan den IJssel, The Netherlands). Nigericin (1 µmol l^{-1}), a K^+/H^+-exchanger, was used to dissipate the pH gradient and valinomycin (1 µmol l^{-1}), a K^+-ionophore, was used to dissipate the membrane potential. Experiments were done in duplicate on separate days, and for each concentration a representative graph is given.

Measurement of ATP concentrations. Experiments were performed in 5 ml of PBS. After 8 min, 0.125 ml of a 20 % wt/vol glucose solution was added to the cell suspension to

give a final concentration of 0.5 % wt/vol. After 23 min of incubation, a 5-g l^{-1} solution of BAC was added to the cell suspension to produce final concentrations ranging from 8 to 25 mg l^{-1}. Samples were taken at appropriate time intervals. Total ATP concentration was measured in 20 µl of sample. External ATP concentration was measured in 20 µl of supernatant fluid from a 100-µl sample (centrifuged at 4 °C for 3 min at 13,000 rpm). Internal ATP concentrations were calculated by subtracting the external ATP concentrations from the total ATP concentrations. The total ATP concentration just before the addition of the disinfectant was taken as 100 %. ATP concentrations were measured in the Lumac biocounter M2500 with the luciferine/luciferase assay as described by the supplier (Lumac, Landgraaf, The Netherlands). Protein concentrations were determined by the bicinchoninic acid method [165] with bovine serum albumin as a standard. Experiments were done in duplicate on separate days, and for each concentration a representative graph is given.

Measurement of acidification. Experiments were performed in 9 ml of 0.2 mmol l^{-1} potassium phosphate buffer (pH 7.2) in a stirred, water-jacketed vessel. The pH was recorded continuously with a pH electrode. A total of 0.1 ml of a 90× concentrated cell suspension (see above) on ice was added. After temperature equilibration (2 min), 0.225 ml of a 20 % wt/vol glucose solution was added to give a final concentration of 0.5 % wt/vol. After 5 min, a 5 g l^{-1} solution of BAC was added to produce final BAC concentrations ranging from 8 to 23 mg l^{-1}. Experiments were done in duplicate on separate days. The average of the two experiments is given.

Measurement of respiration. Experiments were performed in 4 ml PBS in a stirred, water-jacketed vessel. The oxygen concentration was recorded continuously with an oxygen-electrode (YSI model 5300 biological oxygen monitor, Yellow Springs Instruments Co. Inc., Yellow Springs, USA). A total of 0.1 ml of a 40× concentrated cell suspension (see above) on ice was added. After temperature equilibration (2 min), 0.1 ml of a 20 % wt/vol glucose solution was added to give a final concentration of 0.5 % wt/vol. After 5 min, a 5 g l^{-1} solution of BAC was added to produce final BAC concentrations ranging from 9 to 31 mg l^{-1}. Experiments were done in duplicate on separate days. The average of the two experiments is given.

RESULTS

In this study the difference in susceptibility between exponential-phase and stationary-phase cells of *L. monocytogenes* to BAC was studied. Based on the growth curve of *L. monocytogenes* in BHI-broth with 0.5 % wt/vol glucose, exponential-phase cells were harvested after 3.5 h in the mid-exponential-phase and stationary-phase cells at 15.5 hours.

Exponential- and stationary-phase cells were exposed for 5 min to concentrations of BAC ranging from 0 to 25 mg l^{-1} (Fig. 2.1A). At 8 mg l^{-1}, BAC was not effective in killing; only a 0.22-log reduction of both stationary-phase cells and exponential-phase cells was

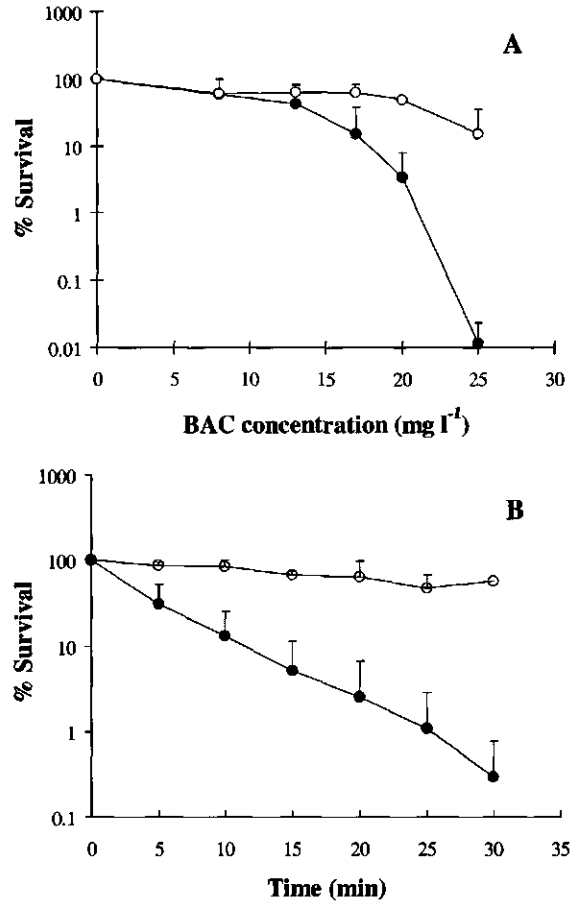

Figure 2.1 Survival of *L. monocytogenes* after exposure to BAC. Cells were exposed for 5 min to different concentrations of BAC (A) or for 30 min to 17 mg l^{-1} BAC (B). Results are shown for exponential-phase cells (●) and stationary-phase cells (○). Error bars are plotted on a logarithmic scale and indicate the standard deviation.

observed. At higher concentrations, BAC caused a dramatic killing of exponential-phase cells: 17 mg l^{-1} of BAC caused a reduction in viable cells of 0.83 log units and 25 mg l^{-1} a reduction of 3.9 log units. In contrast, 17 mg l^{-1} of BAC reduced the number of viable stationary-phase cells only by 0.20 log units and 25 mg l^{-1} caused a reduction of 0.81 log units. Thus, at a concentration of 25 mg l^{-1}, stationary-phase cells were at least a 1,000 times more resistant to BAC than exponential-phase cells.

Exponential- and stationary-phase cells were exposed to a concentration of 17 mg l^{-1} of BAC for 30 min (Fig. 2.1B). At all time points stationary-phase cells of *L. monocytogenes* survived exposure to 17 mg l^{-1} of BAC better than exponential-phase cells. The difference in resistance increased over time. After 30 min treatment the number of viable stationary-phase cells had

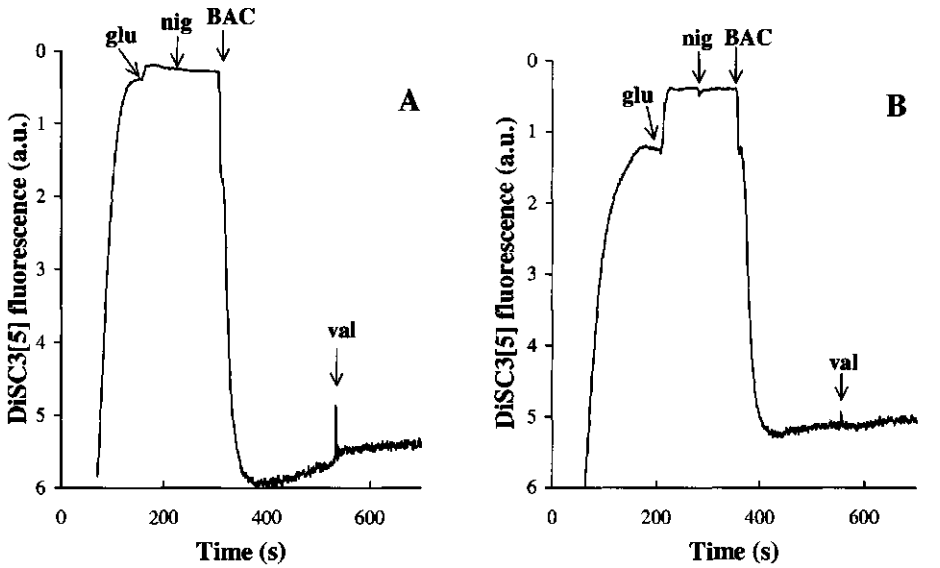

Figure 2.2 Effect of BAC on the membrane potential of *L. monocytogenes*. Assays were performed with exponential-phase cells (A) and stationary-phase cells (B) with the use of $DISC_3[5]$. At the times indicated by arrows the following substances were added: glucose (glu), nigericin (nig), BAC (8 mg l^{-1}) and valinomycin (val). a.u. indicates arbitrary units.

decreased by only 0.25 log units, whereas the number of viable exponential-phase cells had decreased by 2.5 log units. Dodd *et al.* [61] proposed that under aerobic conditions biocides have a secondary killing effect on bacteria which results from an imbalance between anabolism and catabolism. This imbalance could cause a burst of oxygen radicals, resulting in additional cell damage. To verify this hypothesis, we analyzed killing of *L. monocytogenes* by BAC in the presence and absence of oxygen after 5-min exposure. No significant influence of oxygen on killing of *L. monocytogenes* could be shown when exponential-phase and stationary-phase cells were exposed to 25 and 30 mg l^{-1} of BAC, respectively (two different concentrations of BAC were used to attempt to achieve a similar amount of survivors for both cell types). Exponential-phase cells showed a 3.0 log reduction in survival aerobically and a 2.5-log reduction anaerobically. For the stationary-phase cells these numbers were 2.0- and 1.2-log reduction, respectively (data not shown).

Additionally, no significant influence of glucose or BHI medium on killing could be shown when *L. monocytogenes* exponential-phase and stationary-phase cells were exposed to 25 and 30 mg l^{-1} of BAC, respectively, for 5 min in PBS, PBS-g, or BHI-g. For exponential-phase cells, the reductions in log units in PBS, PBS-g and BHI-g were 4.0, 4.3 and 3.2, respectively. For stationary-phase cells, the log reductions were 0.81, 0.68 and 0.85, respectively (data not shown).

In addition to killing experiments the dose-response relationship of *L. monocytogenes*

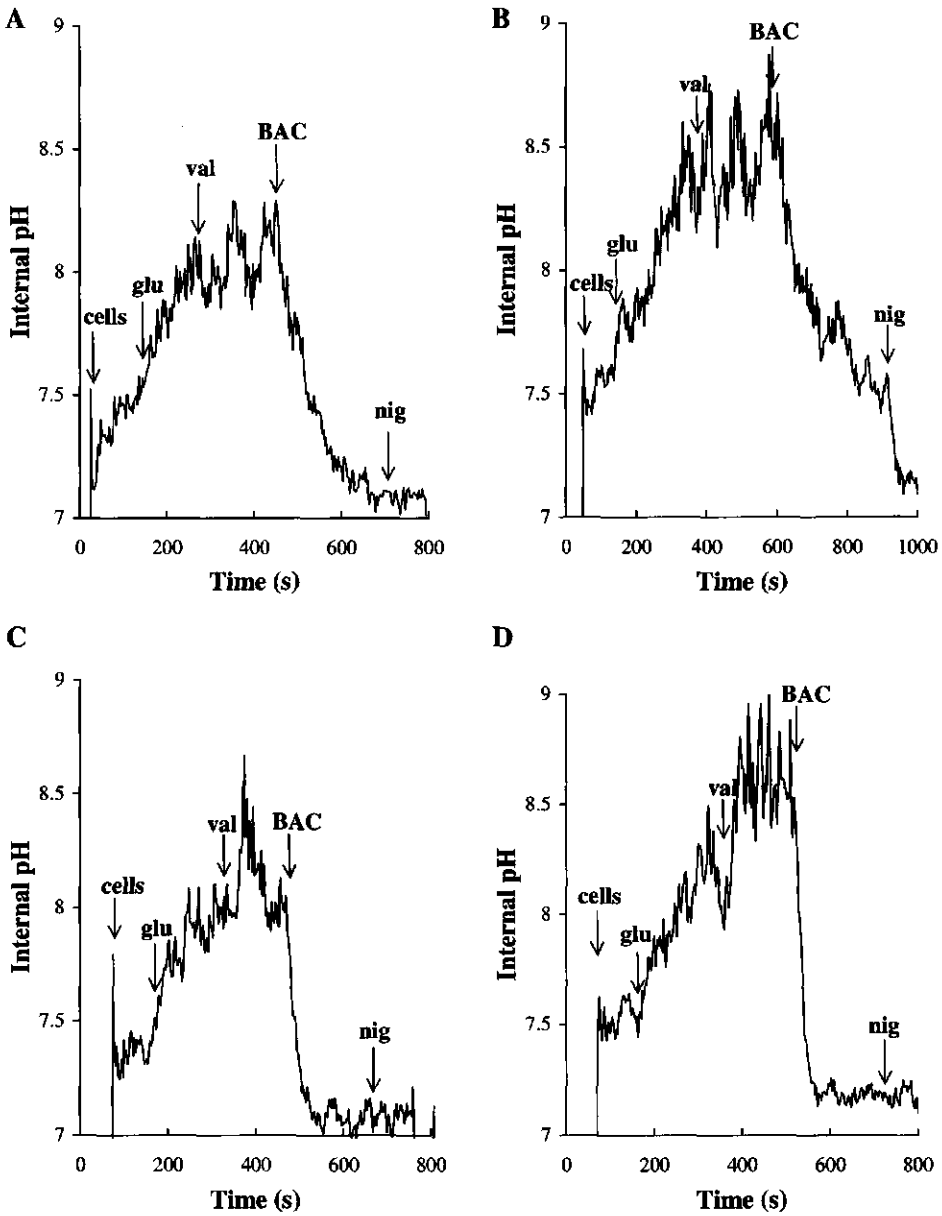

Figure 2.3 Effect of BAC on the intracellular pH of *L. monocytogenes*. Assays were performed with exponential-phase cells (A, C) and stationary-phase cells (B, D). At the times indicated by arrows, the following substances were added: cells labeled with CFSE (cells), glucose (glu), valinomycin (val), BAC and nigericin (nig). BAC was added to concentrations of 8 mg l^{-1} (A, B) and 17 mg l^{-1} (C, D).

to BAC for a variety of energetic parameters was established. The effect of BAC on the membrane potential in exponential- and stationary-phase cells was determined. A total of 8 mg l^{-1} was used as the starting concentration, because only a 0.22-log reduction was observed

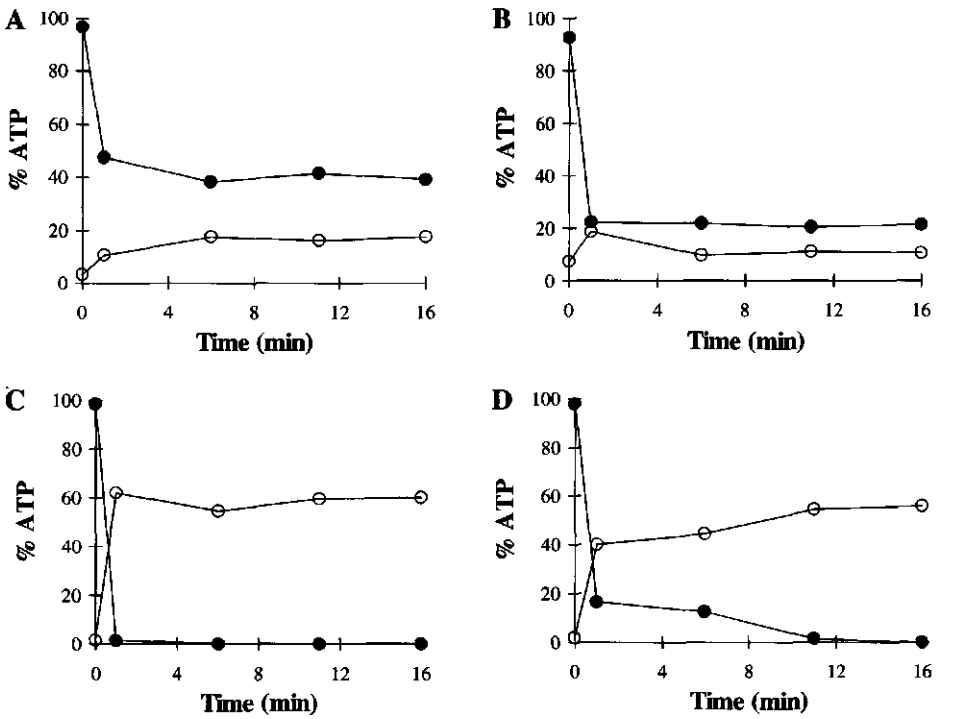

Figure 2.4 Effect of benzalkonium chloride (BAC) on cytoplasmic (●) and extracellular (○) ATP levels for *L. monocytogenes* exponential-phase cells (A, C) and stationary-phase cells (B, D). BAC was added to concentrations of 8 mg l^{-1} (A, B), 20 mg l^{-1} (C) and 25 mg l^{-1} (D). The total amount of ATP just before the addition of BAC was 100 %. For exponential-phase cells and for stationary-phase cells 100 % ATP was 0.56 and 0.52 µmol ATP (mg of protein)$^{-1}$ respectively.

at this concentration and, therefore, there were no major effects expected on the membrane potential. The membrane potential of both exponential- and stationary-phase cells increased slightly on addition of glucose, but did not increase further on addition of nigericin (figs 2.2A and 2.2B). On addition of 8 mg l^{-1} of BAC, exponential- and stationary-phase cells showed similar behavior. The membrane potential was rapidly and completely dissipated since addition of valinomycin did not result in a further reduction of the membrane potential. Without addition of BAC the membrane potential was not affected for at least 5 min and only after addition of valinomycin was it completely dissipated (data not shown).

The internal pH of both exponential- and stationary-phase cells increased on addition of glucose and even further on addition of valinomycin, since the dissipation of the membrane potential is compensated for by an increase of the pH gradient. On addition of 8 mg l^{-1} of BAC the pH gradient decreased slowly in both exponential- and stationary-phase cells. After 4 min the pH gradient was completely dissipated in exponential-phase cells (Fig. 2.3A), since the addition of nigericin did not result in a further reduction of the internal pH. In stationary-phase cells, the pH gradient was almost completely dissipated after 6 min (Fig.

2.3B). At the concentration of 17 mg l^{-1} of BAC, exponential- and stationary-phase cells showed similar behavior. The pH gradient was rapidly and completely dissipated after 1 min (figs 2.3C and 2.3D). In control cells without BAC the internal pH was not affected for at least 5 min and only after addition of nigericin was it completely dissipated (data not shown).

On addition of glucose, the amount of intracellular ATP increased for both exponential- and stationary-phase cells (data not shown). On addition of 8 mg l^{-1} of BAC to glucose-energized cells, exponential- and stationary-phase cells showed similar behavior. At this concentration BAC caused a substantial decrease in internal ATP in both exponential- and stationary-phase cells, but only minor leakage of ATP (figs 2.4A and 2.4B). At higher concentrations, BAC caused complete loss of ATP because of leakage and hydrolysis in both types of cells, but for stationary-phase cells a higher concentration of BAC was needed than for exponential-phase cells. In exponential-phase cells, 20 mg l^{-1} of BAC caused complete loss of ATP after 5 min (Fig. 2.4C). The less sensitive stationary-phase cells were exposed to a higher concentration (25 mg l^{-1}) of BAC. This caused complete loss of ATP, but only after 16 min (Fig. 2.4D).

L. monocytogenes can metabolize glucose to lactic acid and acetic acid resulting in acidification of the medium. Acidification experiments showed that stationary-phase cells were more resistant to inhibition by BAC than exponential-phase cells. BAC completely inhibited acidification at a concentration of 16 mg l^{-1} in exponential-phase cells whereas stationary-phase cells were not inhibited at this concentration. At the concentration of 23 mg l^{-1} acidification was completely inhibited in stationary-phase cells (data not shown). Additionally, the effect of BAC on respiration was investigated. BAC completely inhibited respiration at concentration of 19 mg l^{-1} in exponential-phase cells, whereas stationary-phase cells still respired at about 30 % of the original rate at this concentration. At the concentration of 31 mg l^{-1} respiration was completely inhibited in stationary-phase cells (data not shown).

DISCUSSION

Biofilm bacteria are a problem in the food industry because of their increased resistance to disinfectants. In this study we showed that stationary-phase cells, used as a model for biofilm cells, are much more resistant to BAC than exponential-phase cells.

The dose-response relationship of *L. monocytogenes* to BAC showed that stationary-phase cells and exponential-phase cells react similarly at a low concentration of BAC (8 mg l^{-1}). This low concentration of BAC completely dissipates both components of the proton motive force: the pH gradient and the membrane potential. This suggests that the membrane is damaged and has become permeable to small ions like H^+ and K^+. However, plate counting reveals that there is only a 0.22-log reduction in cell survival, and, therefore, this minor membrane damage is apparently reversible. At higher concentrations, other cell functions start to collapse and differences between stationary- and exponential-phase cells become

apparent. Acidification and respiration are completely inhibited and all residual intracellular ATP leaks out of the cell. For exponential-phase cells, a lower concentration of BAC (at least 17 mg l^{-1}) is needed to produce this collapse than for stationary-phase cells (at least 27 mg l^{-1}). At these concentrations, the reduction in survival is more than 1 log unit. Thus, the cell functions that are disrupted at higher concentrations of BAC give a better indication of killing than dissipation of the proton motive force does. In particular the leakage of ATP, which results in the depletion of intracellular ATP, seems to give a good indication of the bactericidal concentration of BAC.

The dissipation of the proton motive force suggests that at low concentrations BAC damages the membrane of *L. monocytogenes* and causes leakage of ions, like K^+ and H^+. At higher concentrations it causes lethal damage through further disruption of the membrane and consequent complete leakage of ATP concomitant with inhibition of respiration and acidification. These results agree with what is generally accepted as the mode of action of quats: alteration of the semi-permeable properties of the cell membrane leads to leakage of metabolites and coenzymes and disturbance in the delicate balance of metabolite concentrations within the cell [135].

Furthermore, we demonstrate that *L. monocytogenes* stationary-phase cells are more resistant to BAC than exponential-phase cells. At high concentrations of BAC (14 mg l^{-1} and higher), stationary-phase cells show enhanced survival, less ATP-leakage, and less inhibition of acidification and respiration than exponential-phase cells. In addition to the previously reported resistance of stationary-phase cells to acid [145] we now show that those cells are also more resistant to membrane active quats. In our experiment we found the difference in resistance between exponential-phase cells and stationary-phase cells at a BAC concentration of 25 mg l^{-1} to be at least 1,000 fold. This indicates that differences in growth phase and stress response are important factors in resistance to BAC for free-living cells.

Our results also indicate that the presence or absence of BHI medium, including energy sources, during short-term exposure to BAC does not influence the killing of *L. monocytogenes* by BAC. Neither does the presence or absence of oxygen during 5 min of killing, dilution and incubation on plates. It is important to know whether these compounds have an additional influence on killing, because in biofilms the distribution of oxygen and nutrients is not even or constant. Our results are in contrast with the suggestion made by Dodd *et al.* [61] who propose that presence of oxygen will enhance killing by biocides by causing an imbalance between catabolism and anabolism, resulting in a burst of free radicals. This would especially be the case in exponentially growing, metabolically active cells. On the other hand, Denyer and Stewart [59] remark that this imbalance may not occur with membrane active compounds that affect the cell by impairing intracellular homeostasis. This may be the case for *L. monocytogenes* exposed to BAC.

Recently, *L. monocytogenes* was shown to produce an alternative σ-factor for RNA-polymerase, σ^B. This σ-factor appeared to be involved in mediating acid resistance of stationary-phase cells and osmotic stress tolerance [23, 182]. For *Bacillus subtilis*, another

Gram-positive organism, it has been reported that σ^B-dependent transcription is activated on entry into stationary-phase and following exposure to growth-limiting conditions and environmental stress [182]. The regulation of both stress response and entry into stationary-phase by the same alternative σ-factor may provide an explanation for the increased resistance of *L. monocytogenes* to quats in the stationary-phase. For Gram-negative bacteria it is known that an alternative σ-factor (rpoS) plays an important role in biofilm formation [2, 136]. Alternative σ-factors, such as σ^B, may also be important in biofilms formed by Gram-positive bacteria, such as *L. monocytogenes*.

In summary, we can conclude that testing of free living cells in different growth phases indicates whether large differences in resistance to disinfectants occur and that this may be useful to predict a part of the resistance of biofilm cells to disinfectants.

ACKNOWLEDGEMENT

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Chapter 3

The effect of the growth phase of *Staphylococcus aureus* on resistance to disinfectants in a suspension test

S.B.I. Luppens, F.M. Rombouts, T. Abee

The influence of growth phase on the resistance of *Staphylococcus aureus* to the surface-active agents benzalkonium chloride and dodecylbenzyl sulphonic acid and the oxidizing agents sodium hypochlorite and hydrogen peroxide was studied. The resistances of cells in different growth phases were compared to those of solid medium cells grown according to the European phase 1 suspension test. Using cells from different growth phases ($\pm 3\times10^7$ CFU ml^{-1}) we found that decline-phase cells were the most resistant cells. However, the decline-phase cell suspension contained more than 90 % dead cells. A 10-fold diluted suspension with a total concentration of cells equal to that of the other cell suspensions still revealed decline-phase cells generally to be the most resistant cell type. However, the resistance was drastically reduced indicating that the large proportion of dead cells provided a significant protection to the viable decline-phase cells. Hydrogen peroxide resistance could be partly explained by the high catalase activity in the dead cell fraction. Exponential-phase cells were less resistant than decline-phase cells, and, surprisingly, stationary-phase cells were the least resistant of the three. Cells grown according to the European phase 1 suspension test were never the most resistant cells. Their survival was 1 to 3 log units less than that of the most resistant cells. These findings show that the solid medium cells currently used are not the most resistant type of cells that can be used.

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INTRODUCTION

The phase 1 suspension test is de first test in a series of tests that a substance has to pass to be approved as a disinfectant for a specific use. In phase 1 suspension tests *Staphylococcus aureus* is the bacterium used to represent the Gram-positive bacteria [10]. In short a phase 1 suspension test consists of the following procedure: Cells that have been grown for a day on solid medium are suspended, and a certain amount of the suspension is added to a solution of the substance to be tested. The number of survivors is determined after different exposure times and compared with a control. The substance passes the test if it has caused survival decrease by more than 5 log units after 60 min [10]. After the phase 1 suspension test, more specific tests follow; these tests determine the effects of interfering substances, simulate real-life conditions, or are real field tests [8, 9, 156].

Some of the concerns about suspension tests are the lack of test reproducibility and the predictive value of laboratory grown cultures for naturally occurring strains [114, 148]. Langsrud and Sundheim [114] showed that pregrowth in the absence of oxygen and the use of spread plates instead of pour plates increase the survival of *S. aureus* after exposure to benzalkonium chloride (BAC) and grapefruit extract. On the other hand, Payne *et al.* [148] showed that clinical isolates were not more resistant than *S. aureus* DSM 799, the strain that is used in suspension tests.

Apart from the nature of the strain, the growth phase could influence the resistance of bacteria. Bacteria that are grown in batch culture go through different growth phases: After the exponential growth phase, in which bacteria are supplied with an excess of nutrients and grow fast come the stationary-phase and decline-phase in which there is a lack of certain nutrients. Bacteria are known to respond to this starvation stress by growth rate reduction and induction of defense mechanisms [71, 93, 110]. As a result, they may become more resistant to other types of stress, such as that caused by disinfectants [78, 109, 128, 155, 187]. Thus, growth phase may have substantial influence on resistance of *S. aureus* to disinfectants, and cells in the stationary-phase or the decline-phase may have higher resistance to disinfectants than the cells used currently in suspension tests. The importance of growth phase has already been recognized for *Pseudomonas aeruginosa*, the representative for the Gram-negative bacteria in suspension tests [77].

This report describes the disinfectant resistances of *S. aureus* DSM 799 cells in different growth phases compared with those of cells grown on solid medium as prescribed by the European phase 1 suspension test. Two surface-active agents (BAC and dodecylbenzyl sulphonic acid (DSA)) and two oxidizing agents (sodium hypochlorite (NaOCl) and hydrogen peroxide (HP)) were tested. The tests revealed that the solid medium cells never are the most resistant to the disinfectants. Implications of this finding for the current suspension test are discussed.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *S. aureus* DSM 799 stock cultures were kept at -80 °C with 25 % wt/vol glycerol added. Cells were grown at 37 °C in two different ways. Following the prescription of the European phase 1 suspension test [10], cells were grown on tryptone soya agar (TSA) for 24 h, and then new TSA was inoculated and incubated for 24 h. Then cells were suspended in peptone physiological salt solution (pps; 1 g of neutralized bacteriological peptone and 8.5 g of NaCl per liter) to an $O.D._{620\ nm}$ corresponding with 1.5×10^8 to 5×10^8 CFU ml^{-1}. These cells will be referred to as solid medium cells. All other cells were grown in tryptone soya broth (TSB). A statically grown overnight culture was used to inoculate 30 ml of fresh medium into a 100 ml Erlenmeyer flask (2 % vol/vol inoculum). This new culture was shaken in a gyrorotatory incubator at 130 rpm. Exponential-phase cells were harvested after 2.5 h, stationary-phase cells after 22 h and decline-phase cells after 7 days. From 20 h on, decline-phase cells were incubated statically. Before use, cells were washed twice with pps. Cells were resuspended to a concentration ranging from 1.5×10^8 to 5×10^8 CFU ml^{-1} in pps. These cells will be referred to as liquid cultured cells. All further experiments and assays were performed at 20 °C.

Chemicals and disinfectants. The disinfectants used in this study were alkyl-benzyl-dimethylammonium chloride, alkyl distribution from C_8H_{17} to $C_{16}H_{33}$ (BAC; Lamers & Pleuger, Den Bosch, The Netherlands), 300 g l^{-1} HP (Merck, Darmstadt, Germany), DSA (a 97 % mixture of isomers; Acros, Geel, Belgium) and NaOCl with 130 g l^{-1} active chlorine (Acros). BAC and DSA were dissolved in demineralized water to a concentration of 50 g l^{-1} and sterilized by passage through a 0.2 µm filter. To increase the performance of the DSA solution, 170 g l^{-1} of phosphoric acid was added to it [65]. For BAC, DSA and NaOCl a 10-fold dilution in demineralized water was prepared from the stock solutions before each experiment and used immediately. TSB, TSA and neutralized bacteriological peptone were obtained from Oxoid (Basingstoke, UK). Glycerol was from Fluka Chemie AG (Buchs, Switzerland). Lecithin from soybeans was from BDH (Poole, England). All other chemicals were from Merck KGaA (Darmstadt, Germany).

Percentage viable cells. Cell suspensions were prepared as described above. The concentration of viable cells was determined by diluting the cell suspension in pps and enumerating by plating on TSA and incubating for 48 h at 37 °C. The total concentration of cells was determined by counting the suspension under a light microscope using a Bürker-Türk counting chamber, with a depth of 0.01 mm at × 1,000 magnification. For each sample 63 squares of 0.0025 mm^2 were counted. The percentage of viable cells was calculated by dividing the concentration of viable cells by the total concentration of cells and multiplying by 100 %. Experiments were performed in quadruplicate.

Killing experiments. Cell suspensions were prepared as described above. Killing experiments were performed according to the prescriptions of the European phase 1 suspension test [10]. In brief, 1 ml of cell suspension and 1 ml of demineralized water were

added to 8 ml of disinfectant. To determine the initial number of cells, 1 ml of cell suspension was added to 9 ml pps. After 5, 10, 15, 30, 45 and 60 min a 1 ml sample was taken and transferred to 9 ml of neutralizer. For DSA this neutralizer was a 0.080 mmol l^{-1} NaOH solution. For all other disinfectants and the control the neutralizer consisted of 10 ml of a 34 g l^{-1} KH_2PO_4 buffer adjusted to pH 7.2 with NaOH, 3 g of lecithin from soy beans, 30 ml Tween 80, 5 g of $Na_2S_2O_3$ and 1 g of L-histidine per liter. After 5 min, an appropriate dilution of the neutralized suspension was made in pps, and the sample was enumerated by plating on TSA immediately after dilution. In the original suspension test pour plates are used, but Langsrud and Sundheim [114] showed that the use of pour plates reduced the number of surviving *S. aureus* cells exposed to BAC significantly. The plates were incubated at 37 °C and the colonies were counted after 48 h.

pH of the medium. Solid medium cells were grown for 24 h at 37 °C on TSA with 4 of ml neutral red solution (1 % in 70 % ethanol) per liter (TSA-n) as a pH indicator. After 24 h growth of *S. aureus* on the plates the color of the TSA-n was compared with other sterile TSA-n whose pH was known to range from 6.8 to 8.0. From this comparison the pH of the TSA surrounding the solid medium cells was estimated. Liquid cultured cells were harvested at the proper time (see above). The pH of filter-sterilized (0.2 µm) supernatant was measured with a PHM240 pH/ion meter (Radiometer, Copenhagen, Denmark). All experiments were performed in triplicate.

Catalase activity. Catalase converts HP to water and oxygen. Experiments to determine the oxygen production rate of catalase from HP were performed in 4 ml phosphate buffered saline (0.2 g of KCl, 0.2 g of KH_2PO_4, 1.5 g of Na_2HPO_4 and 8.0 g of NaCl per liter, adjusted to pH 7.2 with HCl) in a stirred, water-jacketed vessel. The dissolved oxygen concentration was recorded continuously with an oxygen-electrode. We measured from 20 % oxygen (air) saturation to 100 % oxygen saturation. First, 0.1 ml cell suspension (see above) was added. After 2 min to allow for temperature equilibration, HP stock solution was added to produce final HP concentrations ranging from 0.75 to 11.25 g l^{-1}. From the resulting oxygen production rates, the HP breakdown rates were calculated, a Michaelis-Menten plot was drawn and the K_m and V_{max} were determined. The K_m values for the different cells ranged from 0.07 to 1.1 mol l^{-1}. Since the highest concentration of HP in our experiment was 0.59 mol l^{-1} we assumed the HP breakdown rate to be first order and in this way we estimated the time required to break down 20 g l^{-1} HP for the CFU present in the killing assays.

RESULTS

Growth curve. In this study exponential-phase cells, stationary-phase cells, decline-phase cells and solid medium cells of *S. aureus* were compared for their susceptibility to disinfectants. Fig. 3.1 shows the growth curve of *S. aureus* in TSB, which was used to determine at what time exponential-, stationary- and decline-phase cells could be harvested.

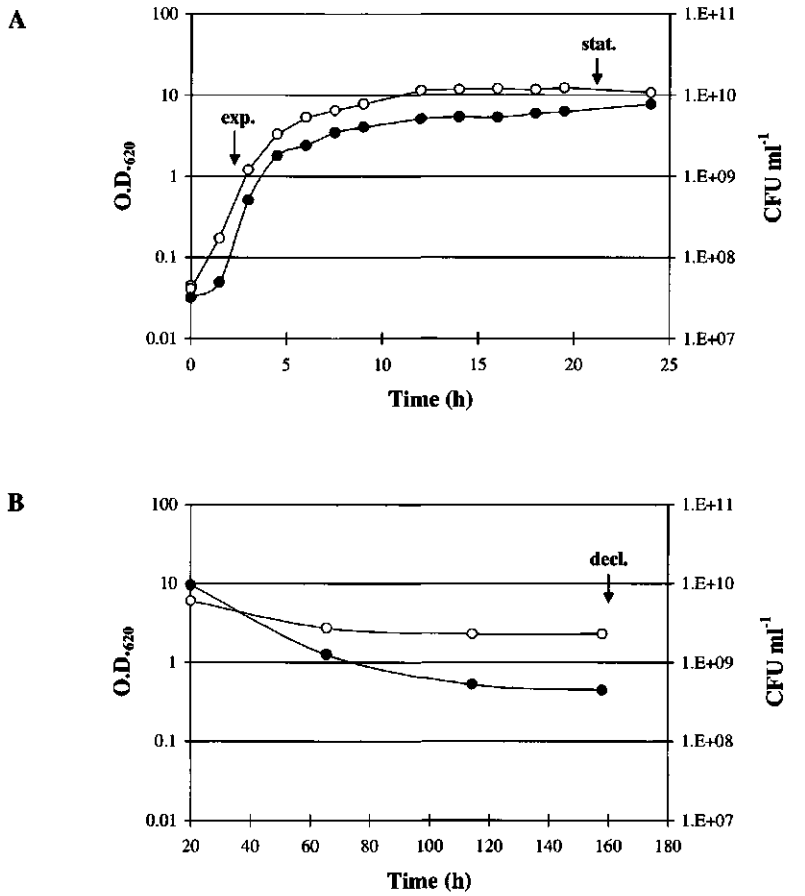

Figure 3.1 Growth curves of *S. aureus* in TSB. Curves for 0 to 24 h (A) and 18 to 158 h (B) are shown. Arrows indicate the times at which exponential-phase cells (exp.), stationary-phase cells (stat.), and decline-phase cells (decl.) were harvested. Both $O.D._{620}$ (○) and CFU ml^{-1} (●) are presented.

After inoculation, *S. aureus* showed a short lag phase of about 1 h, an exponential growth phase from 2 to 5 h after inoculation, a stationary-phase from 10 to 24 h (Fig. 3.1A) and a decline-phase starting after 24 h and reaching a new plateau after 7 days (Fig. 3.1B). We chose to harvest exponential-phase cells in the mid-exponential-phase at 2.5 h, stationary-phase cells at 22 h and decline-phase cells at 7 days (indicated by arrows in Fig. 3.1).

Notable in Fig. 3.1B is that there are many more dying cells (indicated by the decline in CFU) than lyzing cells (indicated by the decline in O.D.) during the decline-phase . Therefore, we determined the percentages of viable cells at the different growth stages (Table 3.1). The percentages viable cells for exponential-phase, stationary-phase and solid medium cells were in the same range: 42 to 86 %. However, the percentage of viable cells in the decline-phase was much lower i.e. 6.4 % of the total number of cells was found on plates.

Table 3.1 Percentages of viable cells in the different cell suspensions used in this study.

Culture type and growth phase	% viable cells[a]
Solid medium	86 ± 16
Liquid medium, exponential phase	42 ± 12
Liquid medium, stationary phase	66 ± 33
Liquid medium, decline phase	6.4 ± 3.7

[a] Values are averages of four measurements and were determined by dividing the ratio of colony forming units by the total number of cells counted in a counting chamber

Thus, the population of cells in the decline-phase is predominantly composed of dead cells (90 to 95 %). Since dead cells may reduce the efficiency of the disinfectant, resistance to the disinfectant may be overestimated. Therefore, we decided to use two suspensions of decline-phase cells: a suspension with the same viable cell concentration as the suspensions of other cell types (see above for preparation) and a suspension with the same total cell concentration as the suspensions of the other cell types (a 10-fold diluted cell suspension of the former).

Killing experiments. A substance passes the European phase 1 test if it has caused survival to decrease by more than 5 log units after 60 min. In our experiments we attempted to obtain this log 5 killing after 60 min for the solid medium cells. Exponential-phase, stationary-phase, decline-phase and solid medium cells were exposed to the surface-active agents BAC and DSA for 60 min (Fig. 3.2). Of cells exposed to 5 mg l^{-1} BAC decline-phase cells were the most resistant, which was obvious already after 10 min (Fig. 3.2A). However, this resistance decreased considerably when the decline-phase cell suspension was diluted 10 times. The diluted decline-phase cells were less resistant than the stationary-phase and exponential-phase cells. Still, all liquid cultured cells were much more resistant to BAC than the solid medium cells. The number of viable solid medium cells was reduced by more than 5 log units within 30 min.

Decline-phase cells were by far the most resistant cells when exposed to 15 mg l^{-1} DSA with 51 mg l^{-1} phosphoric acid, even after dilution (Fig. 3.2B). The survival curves of both decline-phase cell suspensions resembled their survival curves after exposure to BAC. This was also the case for solid medium cells. Their decrease in survival was again more than 5 log units after 30 min. Exponential-phase and stationary-phase cells were even more susceptible to DSA than were solid medium cells: a reduction of more than 5 log units was achieved after 10 min. Exposure of all cell types to 51 mg l^{-1} phosphoric acid alone did not result in a reduction of viable counts after 60 min, except for the stationary-phase cells that showed a 25 % reduction (data not shown).

All cell types were exposed for 60 min to the oxidizing agents NaOCl and HP (Fig. 3.3). For cells exposed to 30 mg l^{-1} NaOCl exponential-phase cells, diluted and undiluted decline-phase cells were the most resistant, but the difference in survival with solid medium

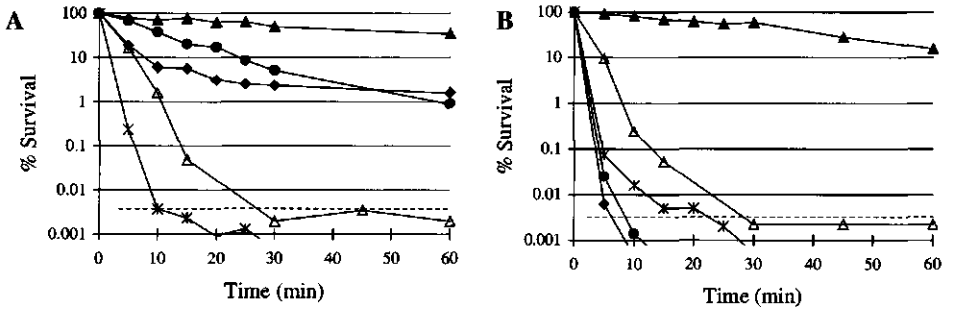

Figure 3.2 Survival of *S. aureus* cells exposed to 5 mg l^{-1} BAC (A) or to 15 mg l^{-1} DSA with 51 mg l^{-1} phosphoric acid (B). Results are shown for exponential-phase cells (●), stationary-phase cells (◆), decline-phase cells (▲), 10-fold-diluted decline-phase cells (△) and solid-medium cells (✱). 100 % survival corresponds with approximately 3×10^7 CFU ml^{-1}. Values are an average of two experiments with less than 1 log unit difference. It should be noted that counts below 0.005 % are not reliable for the 10-fold-diluted decline-phase cells.

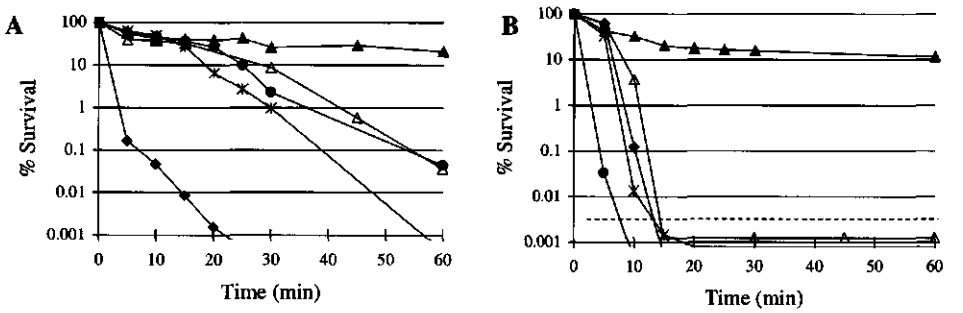

Figure 3.3 Survival of *S. aureus* cells exposed to 30 mg l^{-1} NaOCl (A) or to 20 g l^{-1} HP (B). Results are shown for exponential-phase cells (●), stationary-phase cells (◆), decline-phase cells (▲), 10-fold-diluted decline-phase cells (△) and solid-medium cells (✱). 100 % survival corresponds with approximately 3×10^7 CFU ml^{-1}. Values are an average of two experiments with less than 1 log unit difference. It should be noted that counts below 0.005 % are not reliable for the 10-fold-diluted decline-phase cells.

cells only started to show only after 20 min exposure (Fig. 3.3A). Stationary-phase cells were the most susceptible: after 30 min their survival had already decreased by more than 5 log units.

Decline-phase cells were the most resistant cells, even after dilution, when exposed to 20 g l^{-1} HP (Fig. 3.3B). This finding is similar to the results for exposure to NaOCl. After 10 minutes, the number of viable decline-phase cells in the diluted suspension had decreased by only 1.3 log units. The other cell types were much more susceptible. Stationary-phase cells showed a reduction of 3 log units, solid medium cells a reduction of 4 log units and

Table 3.2 pH of the surrounding culture medium for the cell types used in this study at the time of harvesting.

Culture type and growth phase	pH of growth medium[a]
Solid medium	7.9 ± 0.14^b
Liquid medium, exponential phase	7.0 ± 0.07^c
Liquid medium, stationary phase	7.8 ± 0.07^c
Liquid medium, decline phase	8.3 ± 0.15^c

[a] Values are averages of three measurements.
[b] Initial pH of medium was 7.0.
[c] Initial pH of medium was 6.8.

Table 3.3 Time necessary for catalase in the cell types used in this study to break down 20 g l^{-1} HP[a].

Culture type and growth phase	Estimated time required to degrade 20 g l^{-1} HP in the reaction mixture (min)
Solid medium	2×10^2-4×10^1
Liquid medium, exponential phase	2×10^2-7×10^1
Liquid medium, stationary phase	9×10^1-3×10^1
Liquid medium, decline phase	2×10^1-7×10^0

[a] The time necessary to break down 20 g l^{-1} HP was estimated for the number of cells present in the killing assays (1.5×10^7 to 5×10^7 CFU ml^{-1}).

exponential-phase cells a reduction of more than 5 log units in the same time interval. Furthermore, the relative susceptibilities of these cell types to one another were completely different from the relative susceptibilities observed with exposure to NaOCl.

pH of the medium. Exposure of cells in various growth phases and conditions to extreme pH values could be responsible for a difference in acid or alkaline pH adaptation of cells. Such an adaptive response might influence cell susceptibility to a disinfectant with low pH, such as DSA with phosphoric acid, or a disinfectant with high pH, such as NaOCl. Therefore, we determined the pH of the surrounding medium for each cell type (Table 3.2) and the pH in the experimental mixture. The pHs in the experimental mixture were 3.5 for DSA with phosphoric acid and 7.6 for NaOCl. For all cell types the pH of the surrounding medium was higher than that of the original medium. For liquid cultured cells the pH increased steadily during growth from pH 6.8 to pH 8.3 in the decline-phase. The medium pHs for stationary-phase and solid medium cells were similar, 7.8 to 7.9. Apparently, only decline-phase cells have been exposed to alkaline pH (pH 8.3), whereas the other cells types were exposed to neutral pH ranging from pH 7.0 to 7.9.

Catalase activity. Differences in the survival rates of cells exposed to HP might be caused by differences in catalase activity. Therefore we estimated the time necessary for the

catalase in the different cell types to convert all HP present in the experimental mixture (Table 3.3). HP degradation times were similar for all cell types, except the decline-phase cells. The time necessary for these cells to convert all HP was at least four times as short as that for any of the other cell types. As mentioned above, the decline-phase cell suspension contained 10 times as many cells as the other suspensions, and the catalase activity of these, predominantly dead cells was most likely responsible for this faster breakdown.

DISCUSSION

This study shows that growth phase has a large influence on the susceptibility of *S. aureus* to disinfectants. Strikingly, for each disinfectant the relative susceptibility of cells in different growth phases varies, even for disinfectants with a similar mechanism of action.

The survival of cells exposed to HP might be influenced by a difference in catalase activity in the different cell types. For exponential-phase, stationary-phase and solid medium cells we found no substantial differences in catalase activity, although exponential-phase cells died more rapidly than the other two cell types. Apparently, exponential-phase cells have a lower inherent resistance to HP than the other two cell types. In undiluted decline-phase cells the catalase breaks down the HP at least four times as fast as it does in the other cell types. This faster breakdown of HP could be caused by the presence of more catalase or more catalase activity in decline-phase cells or by catalase that is still active in the dead cells that are present in abundance (90 to 95 %) in the undiluted decline-phase cell suspension. The relatively very high resistance of undiluted decline-phase cells may be explained by the short HP breakdown time, which results in reduced exposure to HP. All HP is broken down well before the end of the 60 min in decline phase cells, whereas it generally takes 60 min or longer for this to happen in other cells. Nevertheless, when a 10-fold-diluted decline-phase cell suspension was used, cell survival was still higher than that of the other cell types. Decline-phase cells apparently have a higher inherent resistance to HP than do other cell types.

Another factor that could influence survival is the pH of the medium surrounding the cells during growth. This external pH could induce acid or alkaline adaptation in cells [71, 72, 145] and thereby influence their survival after exposure to disinfectants with a low pH (DSA with phosphoric acid) or a high pH (NaOCl). However, we found that most of the different cell types of *S. aureus* used in our experiments were surrounded by medium with pH values ranging from 7.0 to 7.9. Therefore, we do not expect acid or alkaline adaptation to be of importance. Whether the high external pH of the medium surrounding the decline-phase cells (pH 8.3) contributes in some way to their increased survival capacity remains to be elucidated.

A factor that did have a large influence on the efficiency of the disinfectants used in this study was the large proportion of dead cells in the decline-phase cell suspension. Such

cells may still react with disinfectants either directly, e.g., by binding membrane active compounds such as BAC or indirectly, e.g., by the contribution of their enzymes such as catalase in the degradation of HP. In this way they may protect the viable cells in the suspension. Our results show that decline-phase cells indeed are more resistant in the presence of these dead cells, which account for more than 90 % of the population. When the cell suspension was diluted to decrease the total number of cells to that of the suspensions of the other cell types, a large drop in resistance was seen. Therefore, if the resistance of cells in different growth phases is tested, the number of viable cells and the number of dead cells in the suspension should be taken into account to allow for a fair comparison between the different cell types. Watson *et al.* [179] have analyzed the stress resistance of *S. aureus* 8325-4 cells in different growth phases and noticed that decline-phase cells also showed the highest resistance to stress. However, in their experimental set-up they did not take the presence of dead cells into account, i.e. only the concentration of viable cells was determined. From their results it appears that the decline-phase cells suspension contains a large fraction of dead cells and the resistance of the viable cells may therefore have been overestimated.

Nevertheless, in our study after a 10-fold dilution, decline-phase cells were still the most resistant cells, except when they were exposed to BAC. Exponential-phase cells were the second most resistant cells and stationary-phase cells were the most susceptible ones. This finding is very striking because it is generally accepted that cells in stationary-phase are more resistant to stress conditions, including exposure to antimicrobial agents, than are exponential-phase cells [78, 109, 122, 128, 145, 155, 187]. Our results are in agreement with those of Watson *et al.* [179] (see above) who observed the HP resistance of *S. aureus* 8325-4 to decrease from decline-phase cells to exponential-phase cells to stationary-phase cells. Cells exposed to acid and heat exhibited similar resistance patterns. Watson *et al.*'s [179] explanation for the sensitivity of stationary-phase cells is based on the biphasic death curves observed for stationary-phase cells. It was proposed that the stationary-phase cells that showed reduced resistance to the treatments were the same cells that would die upon long-term starvation. This proposal is based on the concept that the cells become committed to survival or death early after entry into the stationary-phase unless they are rescued by provision of nutrients. In our experiments biphasic death patterns were also observed for stationary-phase cells exposed to NaOCl and BAC. When exposed to the other disinfectants, stationary-phase cells die too rapidly for any conclusions to be drawn.

The most important finding in this study is the lower resistance of solid medium cells compared with that of liquid cultured cells. After 60 min, solid medium cells never had the highest resistance to any of the disinfectants used. In all cases the resistance of one or more liquid cultured cell types was 10 to 1,000-fold higher. Even after 5 min exposure, solid medium cells showed a survival rate that was the same as or lower than those of other cell types. Five minutes is the exposure time used for other types of suspension tests, such as the suspension test used in phase 2 step 1 of the European disinfectant tests [9]. It can be concluded that the current suspension tests underestimate the phenotypic resistance of *S.*

aureus cells to disinfectants. Therefore, for suspension tests it would be advisable to use cells other cell than the solid medium cells that are currently used.

In summary, we show that differences in resistance to disinfectants as large as 1,000-fold are possible for *S. aureus* grown in liquid medium and on solid medium and the solid-medium cells are not the most resistant cells that can be used in a disinfectant test. Our further research will focus on another factor that influences the resistance of cells to disinfectants: growth in a biofilm.

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Chapter 4

Viability assessment of disinfectant stressed *Listeria monocytogenes* by flow cytometry and plate counting

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In this study we compared fluorescent labeling methods with plate counting as indicators for viability of *Listeria monocytogenes* cells exposed to benzalkonium chloride (BAC) and hydrogen peroxide (HP). Furthermore, we studied the influence of growth phase on fluorescent labeling. The fluorescence methods used were labeling with CTC (dehydrogenase activity), labeling with TOTO (membrane impermeant probe) and assessment of the maintenance of a pH-gradient in a low pH buffer after labeling with the pH-sensitive probe CFSE (pH_{in}-method). Growth phase influenced fluorescent labeling, but not to such an extent that it influenced the way in which the distinction between viable and non-viable cells was made. The viability of cells exposed to BAC determined with plate counts correlated well with CTC-labeling and TOTO-exclusion. The viability of BAC exposed and HP exposed cells determined by plate counts did not correlate quantitatively with the pH_{in}-method. However, this method gave a good indication of viability, sub-lethal damage and cell death. CTC-labeling and TOTO-exclusion did not correlate with viability of HP exposed cells determined by plate counts. Notably, at conditions that 0.2 % of the HP-stressed cells were viable according to plate counts, more than 92 % of the cells were classified viable based on exclusion of TOTO. In conclusion, flow cytometry can be used as a rapid alternative for viability assessment by plate counting provided the proper fluorescent probes are selected.

Submitted for publication

INTRODUCTION

Listeria monocytogenes is a ubiquitous Gram-positive food borne pathogen [68, 69, 87]. It is able to attach to and form biofilms on the types of surfaces present in food industry and in households [27, 74, 85, 105, 111, 126, 127, 130, 157]. Attached and biofilm *L. monocytogenes* cells are more resistant to disinfectants than are free-living cells [74, 139, 157]. Consequently, these cells are more difficult to eradicate, leaving a hazard of food contamination. Therefore, it is necessary to quantitatively assess the viability of these attached and biofilm cells after exposure to disinfectants.

The traditional method to determine viability of bacteria is plate counting. This method is based on the reproductive capacity of cells. However, this method has some disadvantages. The plate count technique requires long incubation times (2 days). Furthermore, for viability assessment of biofilm cells, the cells have to be removed from the surface for analysis. Additionally, several studies indicate that cells can be metabolically active while they are incapable of the cellular division required to form a colony on a plate. This is also known as a viable but non culturable (VBNC) state [32, 44, 48, 102, 120] or better: an active but non culturable state (ANC) [100]. In the case of disinfectants this may lead to the overestimation of the efficacy of the disinfectant.

An alternative method to determine viability is labeling with fluorescent probes. These probes indicate if a cell possesses other physiological characteristics required for a cell to be viable, such as membrane integrity, enzyme activity and energy production [34, 100]. Fluorescent probes can be used directly to assess viability of attached cells when their use is combined with fluorescence microscopy (thin layers) [40, 130, 131, 190] or confocal scanning laser microscopy (thick layers) [83, 133, 177]. Fluorescent labeling can also be combined with flow cytometry (FCM). The advantage of such a method is viability assessment can be done in 0.5 to 2 hours. FCM is a technique for individual cell analysis and it has been applied earlier to analyze heterogeneous populations such as starved cells [179] and attached cells [184] and may be used for biofilm cell analysis. Fluorescent labeling is influenced by the growth phase of bacteria [31, 32, 48, 96, 163, 169, 184]. As cells in biofilms face nutrient gradients [26, 57, 188] and are therefore in different growth phases or have differing growth rates [167, 188] we first tested the suitability of fluorescent probes for viability assessment of free-living cells in different growth phases after exposure to disinfectants. The disinfectants that were used in this study were BAC and HP. BAC is a membrane active compound and HP is an oxidizing agent. They are used as disinfectants in the food industry [149, 186].

In this study we tested three fluorescent probes. The first, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) is used to measure dehydrogenase activity associated with respiration [103, 164]. CTC is not fluorescent, but is converted by dehydrogenases into an insoluble red fluorescent compound (CTC-formazan), which accumulates inside the cell. CTC has been used to assess the viability of *L. monocytogenes* after heat stress [31] and to

study its survival in seawater [36]. The second fluorescent probe TOTO-1 iodide (TOTO) is green fluorescent and impermeant to live cells. It has a high affinity for DNA and its fluorescence is greatly enhanced (often over a 1,000-fold) when it binds to DNA [38]. It has been used to assess membrane integrity of *Lactococcus lactis* after exposure to detergents [33] and to assess viability of several lactic acid bacteria after exposure to bile salts, heat and acid [38]. Other DNA-binding membrane impermeant probes are SYTOX Green and Propidium Iodide (PI) [38]. The third fluorescent probe 5-(and 6)-carboxyfluorescein succinimidyl ester (CFSE) is green fluorescent and has been used to measure the internal pH of several bacteria including *L. monocytogenes*, *L. lactis*, *Bacillus subtilis*, *Escherichia coli*, *Listeria innocua* and *Clavibacter michiganensis* [35, 45, 122]. In this study CFSE was used to assess the ability of the cells to maintain a pH gradient in a low pH buffer. CFSE is brought into the cell as CFDA,SE and converted to CFSE if esterase activity is present. The succinimidyl group of CFSE is thought to form conjugates with aliphatic amines, which prevents CFSE extrusion from the cell [35]. Esterase activity is frequently used as a viability indicator [175].

The primary aim of this study was to compare fluorescent labeling methods with plate counting as indicator for viability of *L. monocytogenes* cells exposed to BAC and HP. Furthermore, we studied if growth phase influences fluorescence labeling and if it is possible to predict if a probe will be a good viability indicator for cells exposed to a certain disinfectant on the basis of the mechanism of action of the disinfectant and the target of the fluorescent probe. These findings may be used to select the most suitable probe(s) for viability assessment of biofilm cells exposed to disinfectants.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *Listeria monocytogenes* Scott A was grown at 30 °C in brain heart infusion (BHI) broth with 0.5 % wt/vol glucose. Stock cultures were kept at -80 °C with 25 % wt/vol glycerol added. A statically grown overnight culture of *L. monocytogenes* was used to inoculate 150 ml (exponential-phase) or 30 ml (stationary-phase) of fresh medium (2 % vol/vol inoculum). This new culture was shaken in a gyrorotatory incubator at 150 rpm in a 1 liter (exponential-phase) or 100 ml (stationary-phase) Erlenmeyer flask. Exponential-phase cells were harvested at an $O.D._{620\ nm}$ of 0.20 (after approximately 3.5 h). Stationary-phase cells were harvested after 15.5 h of growth. Before use, cells were washed once by centrifuging for 10 min at 2620 × *g* and resuspending in phosphate buffered saline (PBS) (0.2 g of KCl, 0.2 g of KH_2PO_4, 1.5 g of Na_2HPO_4 and 8.0 g of NaCl per liter, adjusted to pH 7.2 with HCl). Cells were resuspended to a concentration ranging from 7×10^8 to 3×10^9 CFU ml^{-1} in PBS.

Chemicals, probes and disinfectants. Alkyl-benzyl-dimethylammonium chloride, alkyl distribution from C_8H_{17} to $C_{16}H_{33}$ (BAC) was obtained from Lamers & Pleuger (Den

Bosch, The Netherlands) and 300 g l^{-1} hydrogen peroxide (HP) stock solution from Merck (Darmstadt Germany). BAC was dissolved in demineralized water to a concentration of 50 g l^{-1} and sterilized by passing through a 0.2 µm filter. Then a 10-fold dilution in demineralized water was prepared from the stock solution before each experiment and used immediately. CTC was obtained from Polysciences Inc. (Warrington, USA), TOTO (Quinolinium, 1-1'-[1,3-propanediylbis[(dimethyliminio) -3,1-propanediyl]]bis[4-[(3- methyl-2(3H)-benzothiazolylidene) methyl]]-, tetraiodide) and CFDA,SE (5- (and 6)-carboxyfluoresceindiacetate succinimidyl ester) were obtained from Molecular probes Europe BV (Leiden, the Netherlands). CFDA,SE is a non-fluorescent esterase substrate yielding the fluorescent CFSE upon hydrolysis. Stock solutions of 0.68 mmol l^{-1} CFDA,SE in acetone, 100 µmol l^{-1} TOTO in dimethyl sulfoxide and (daily) 5 mmol l^{-1} CTC with 50 mmol l^{-1} glucose in MQ water were prepared. BHI was from Difco (Sparks, USA), the ionophores nigericin and valinomycin were from Sigma Chemical Co. (St. Louis, USA) and glycerol was from Fluka Chemie AG (Buchs, Switzerland). All other chemicals were from Merck KGaA.

Exposure to disinfectants. Cell suspensions were prepared as described above. Killing experiments were performed with 1 ml of cell suspension. BAC (17 to 57 mg l^{-1} final concentration) or HP (0.68 to 1.2 mol l^{-1} final concentration) were added to the cell suspension and mixed. Five minutes after addition the suspension was diluted ten times in PBS, centrifuged for 10 min at 2420 × *g*, the supernatant was discarded and the pellet was resuspended in 1 ml fresh PBS. A control was treated identically, except for the addition of disinfectant. HP exposure experiments were done in duplicate and repeated on a different day. Most BAC exposure experiments were done in duplicate. The statistical test used to analyze the data was a paired students t-test with two tailed distribution and a 0.05 confidence level. The null hypothesis was that there was no significant difference between the viability determined by plate counting on the one hand and each of the fluorescence methods on the other hand.

Measurement of culturability. Appropriate dilutions of the untreated and exposed samples were made immediately in PBS and cells were enumerated by spiral plating in duplicate on BHI-agar immediately after dilution. The plates were incubated at 30 °C and the colonies were counted after two days.

Fluorescence labeling and flow cytometry. For measurement of respiratory dehydrogenase activity samples were diluted ten times in the CTC stock solution to reach a low concentration of phosphate (1.2 mmol l^{-1}) [153] and optimal concentrations of 4.5 mmol l^{-1} CTC [31, 103, 153] and 45 mmol l^{-1} glucose [31], and incubated for 45 min. For measurement of the membrane integrity untreated and exposed samples were incubated with 1 µmol l^{-1} TOTO for 15 min, washed once with PBS and resuspended in 50 mmol l^{-1} potassium phosphate (KPi) buffer adjusted to pH 7.2. CTC and TOTO labeled cell suspensions were kept on ice for not more than 30 minutes. For measurement of maintenance of a pH gradient untreated and exposed samples were incubated with 17 µmol l^{-1} CFDA,SE for 15 min, washed once with PBS, incubated in PBS with 27 mmol l^{-1} glucose for 15 min,

washed once with PBS, resuspended in a ten times larger volume of KPi buffer pH 5.5 with 27 mmol l^{-1} glucose, incubated for 20 min and measured immediately. In addition, untreated cells were resuspended in KPi buffer pH 7.2. Other untreated cells were suspended in KPi buffer of pH 5.5 or pH 7.2 to which 1 µmol l^{-1} nigericin (a K^+/H^+-exchanger) and 1 µmol l^{-1} valinomycin (a K^+-ionophore) were added to dissipate the pH gradient and the membrane potential of the cells, respectively.

Flow cytometric analyses were performed on a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, USA) equipped with a 15-mW, 488-nm, air-cooled argon ion laser. Cell samples were diluted to approximately 5×10^6 cells per ml and measured at the low flow rate, corresponding to 750 to 2500 cells per second. Forward scatter (FSC), side scatter (SSC), and three fluorescent signals were measured. A band pass filter of 530 nm (515 to 545 nm) was used to collect green fluorescence (FL1), a band pas filter of 585 nm (564 to 606 nm) was used to collect yellow-orange fluorescence (FL2) and a long-pass filter of 670 nm was used to collect red fluorescence (FL3). FSC was collected with a diode detector. SSC and FL1 to FL3 were collected with photomultiplier tubes. All signals were collected with the use of logarithmic amplification. SSC was used to discriminate bacteria from background. For all samples 5,000 or 10,000 cells were analyzed. FL1 was used for detection of TOTO, FL3 for CTC and the ratio FL1/FL2 of CFSE was taken as a measure for the internal pH [45]. Data were analyzed with the help of FCSPress program (Ray Hicks, [http://www.fcspress.com/]) and the WinMDI program (version 2.8; Joseph Trotter, John Curtin School of Medical Research, Canberra, Australia [http://jcsmr.anu.edu.au]).

Percentage viable cells. Cell suspensions were prepared as described above. The concentration of viable cells was determined by diluting the cell suspension in PBS and enumerating by spiral plating on BHI and incubating for 48 h at 30 °C. The total concentration of cells was determined by counting the suspension under a phase contrast microscope using a Bürker-Türk counting chamber, with a depth of 0.01 mm at a 1,000 times magnification. For each sample 63 squares of 0.0025 mm^2 were counted. The percentage of viable cells was calculated by dividing the concentration of viable cells by the total concentration of cells and multiplying by 100 %. Experiments were done in quadruplicate.

RESULTS

Viability methods. In this study three fluorescent labeling methods were compared with plate counting as an indicator for viability. Figs 4.1, 4.2 and 4.3 show how viable cells were distinguished from non-viable cells with the different fluorescence methods.

Fig. 4.1 shows BAC exposed and untreated exponential-phase cells labeled with CTC. Two populations could be distinguished (inset). One population had high fluorescence

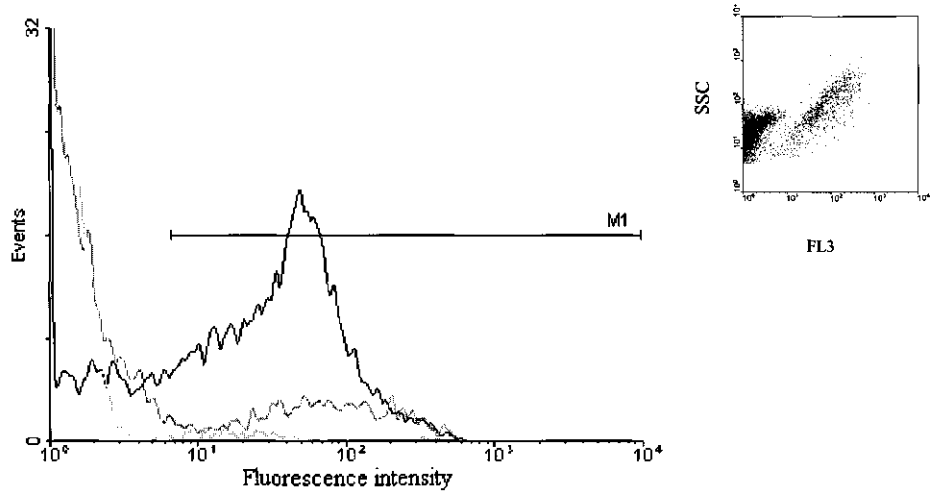

Figure 4.1 Effect of BAC on CTC staining (FL3 fluorescence intensity) of exponential-phase *L. monocytogenes* cells. Results are shown for untreated cells (black line), cells exposed to 17 mg l^{-1} (dark gray line) and 40 mg l^{-1} (light gray line) of BAC. M1 is the region with viable cells. The inset shows a dot plot of FL3 and SSC of cells exposed to 17 mg l^{-1} BAC.

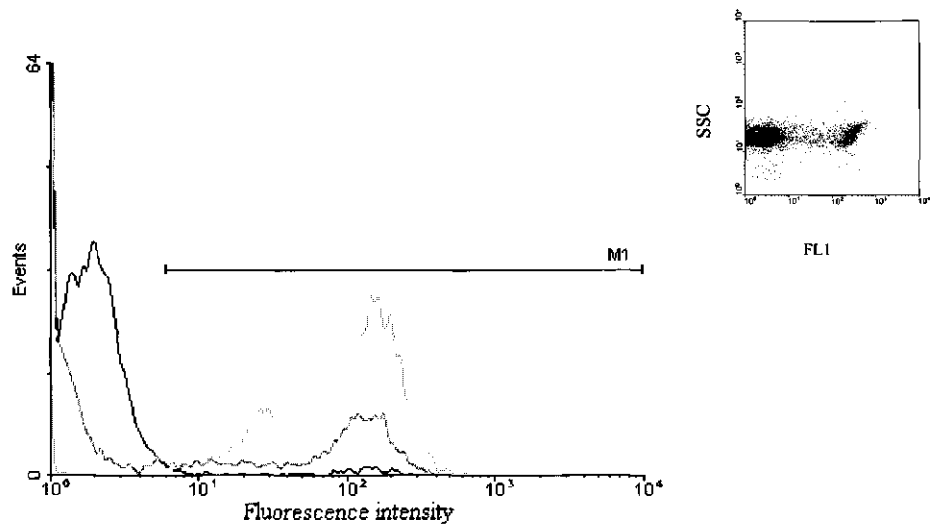

Figure 4.2 Effect of BAC on TOTO staining (FL1 fluorescence intensity) of stationary-phase *L. monocytogenes* cells. Results are shown for untreated cells (black line), cells exposed to 24 mg l^{-1} (dark gray line) and 57 mg l^{-1} (light gray line) of BAC. M1 is the region with non-viable cells. The inset shows a dot plot of FL1 and SSC of cells exposed to 24 mg l^{-1} BAC.

were used to set the region of viable cells as shown in the histogram in Fig. 4.1. This histogram of FL3 against the number of cells demonstrates that not all untreated cells were labeled. Furthermore it shows that the population with respiratory activity decreased when

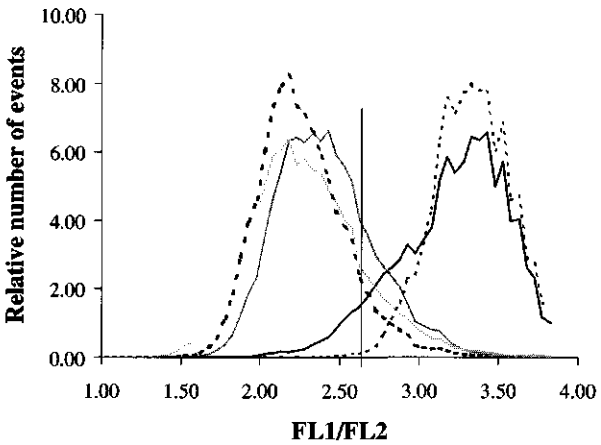

Figure 4.3 Distribution of the FL1/FL2 ratio classes of *L. monocytogenes* stationary-phase cells stained with CFSE and exposed to pH 5.5 with glucose assessed by flow cytometry (pH_{in} method). Results are shown for the untreated cells (black line), cells exposed to 0.68 mol l^{-1} (dark gray line) and 1.1 mol l^{-1} (light gray line) of HP. In addition results are shown for uncoupler treated cells at pH 5.5 (thick dotted line) and pH 7.2 (thin dotted line) in which the pH-gradient was dissipated. Cells to the right of the vertical line are viable.

against the number of cells demonstrates that not all untreated cells were labeled. Furthermore, it shows that the population with respiratory activity decreased when the concentration of BAC increased. At the high BAC concentration (40 mg l^{-1}) only a few cells had respiratory activity (3.8 %).

Fig. 4.2 demonstrates that TOTO labeled cells could clearly be distinguished from non-labeled cells. This is apparent from both the SSC-FL1 dot-plot (inset) and the FL1 histogram. The population with the low fluorescence intensity represents the cells with intact membranes. The population with the high fluorescence has compromised membranes. Practically all untreated cells had intact membranes and the population with intact membranes decreased when the concentration of BAC increased.

Fig. 4.3 shows the FL1/FL2 ratio distribution, which is a measure for internal pH (pH_{in}), in HP exposed cells and untreated cells incubated for 20 min in pH 5.5. This exposure to low pH was not lethal for untreated cells (data not shown). In contrast to the experiments with CTC and TOTO, different fluorescent subpopulations could not be detected. We decided to set the region with non-viable cells on the basis of the percentage non-viable cells initially determined by TOTO labeling. Most of the untreated cells could maintain an internal pH of 7.2. In cells that were exposed to a low concentration of HP (0.68 mol l^{-1}) the ability to maintain a pH-gradient was greatly reduced. Cells exposed to a high concentration of HP (1.12 mol l^{-1}) were unable to maintain a pH gradient.

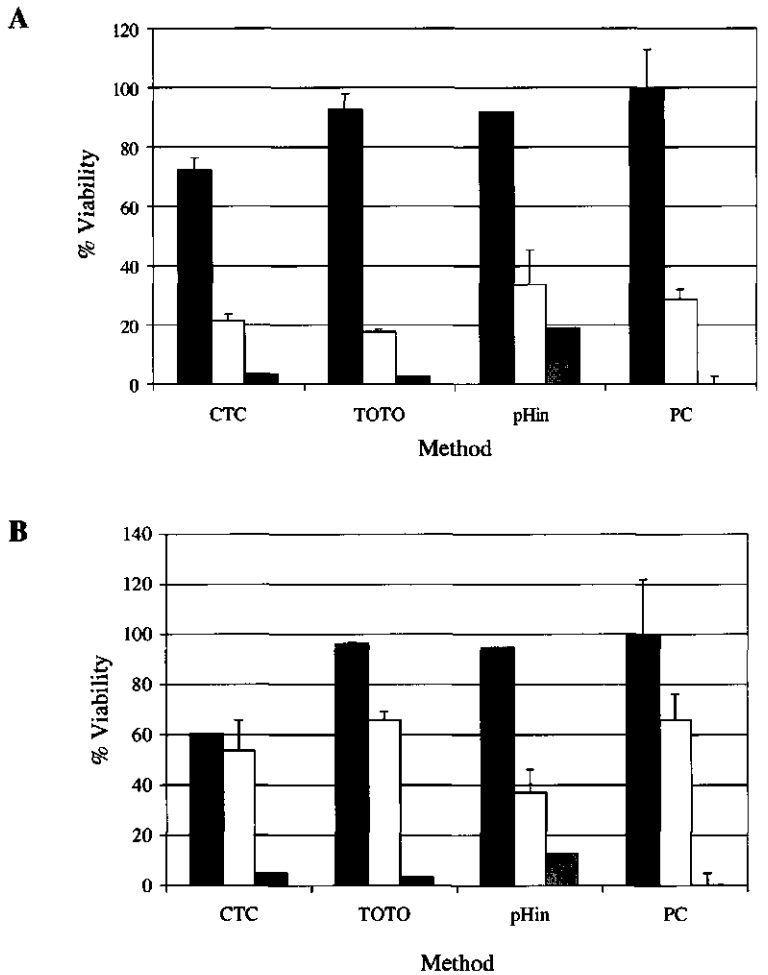

Figure 4.4 Percentage viability of *L. monocytogenes* exponential-phase (A) and stationary-phase (B) cells determined with different methods (PC = plate counting). Results are shown for the untreated cells (black bars) and cells exposed to a low concentration (white bars) and a high concentration (gray bars) of BAC. For exponential-phase cells these concentrations were 17 mg l^{-1} and 40 mg l^{-1}, respectively, for stationary-phase cells 24 mg l^{-1} and 57 mg l^{-1}. Error bars show the standard deviation. 100 % = 6.6×10^{8} CFU ml^{-1} for exponential-phase cells and 2.2×10^{9} CFU ml^{-1} for stationary-phase cells

For the plate counting method we checked the percentage viability of the untreated cells. For exponential-phase cells viability was 125 % ± 86 % and for stationary-phase cells viability was 99 % ± 25 %. Thus, the plate count viability for all untreated cells was set at a 100 % (see figs 4.4 and 4.5).

Exposure to BAC. Fig. 4.4 contains an overview of the results for all four methods that were used to determine viability of *L. monocytogenes* cells exposed to BAC. Stationary-

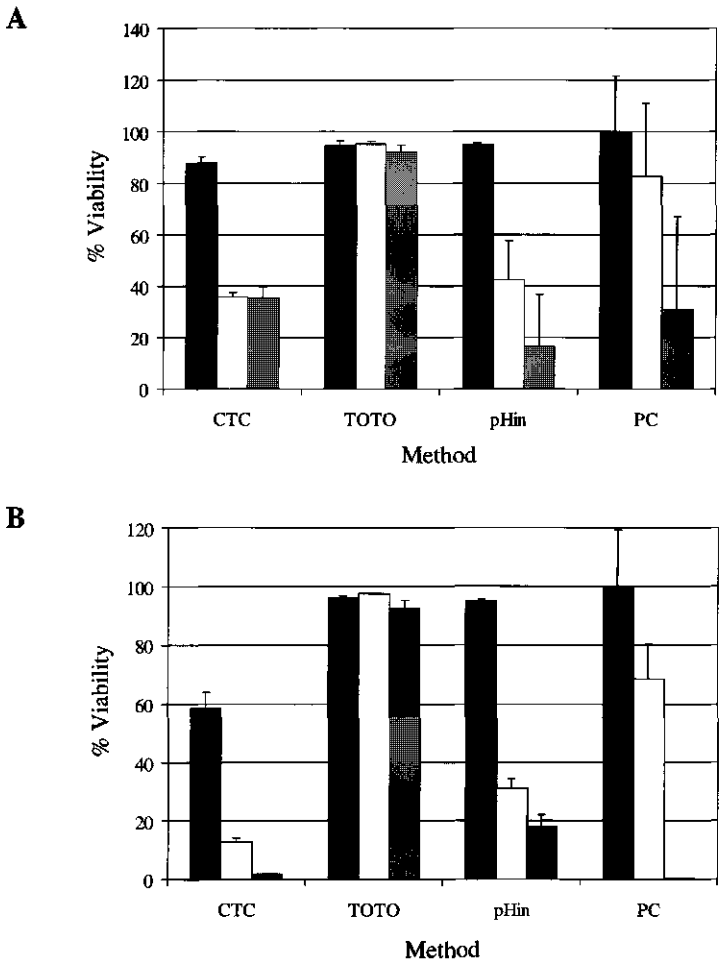

Figure 4.5 Percentage viability of *L. monocytogenes* exponential-phase (A) and stationary-phase (B) cells determined with different methods (PC = plate counting). Results are shown for the untreated cells (black bars) and cells exposed to a low concentration (white bars) and a high concentration (gray bars) of HP. For exponential-phase cells these concentrations were 0.97 mol l^{-1} and 1.2 mol l^{-1}, respectively, for stationary-phase cells 0.68 mol l^{-1} and 1.1 mol l^{-1}. Error bars show the standard deviation. 100 % = 2.7×10^{9} CFU ml^{-1} for exponential-phase cells and 2.5×10^{9} CFU ml^{-1} for stationary-phase cells

phase cells were more resistant to BAC than were exponential-phase cells. **CTC.** CTC labeling of cells in both growth phases correlated well with plate counts, except for the labeling of untreated cells. Especially in stationary-phase cells labeling of control cells was very low (60 %). Furthermore, fluorescence intensity of untreated cells was higher in stationary-phase cells than in exponential-phase cells (data not shown). **TOTO.** TOTO exclusion by cells in both growth phases correlated well with plate counts. Fluorescence

intensity was higher in stationary-phase cells than in exponential-phase cells (data not shown). **CFSE/pH_{in}.** All cells were labeled with CFSE (confirmed with fluorescence microscopy, results not shown) even after exposure to the high concentration of BAC, although after exposure to BAC the fluorescence intensity was about 0.5 log units lower than without exposure. These results indicate that esterase activity was still present in all samples. The pH_{in}-method correlated well with plate counts for control cells and exponential-phase cells exposed to the low BAC concentration, but not with plate counts for the other BAC exposed cells.

Exposure to HP. Fig. 4.5 contains an overview of the results for all four methods that were used to determine viability of *L. monocytogenes* cells exposed to HP. Notably, exponential-phase cells were more resistant to HP than stationary-phase cells. **CTC.** Not all untreated cells were labeled with CTC; in stationary-phase cells even less than 60 % was labeled. Of the exponential-phase cells exposed to HP about 35 % were labeled with CTC, regardless of the HP concentration. Only at the low HP concentration there was a significant difference between CTC labeling and plate counts. When less than 0.5 % of the exponential-phase cells were viable according to plate counts, still 29 % of the cells were labeled by CTC (data not shown). Viability indicated by CTC-labeling of stationary-phase cells was significantly different from plate counts for the untreated cells and cells exposed to HP. These results may suggest that HP interfered in some way with CTC-labeling, but labeling was not a problem when the small trace of HP that could be present after washing of HP exposed cells was added to viable cells just after the beginning of CTC-labeling (data not shown). **TOTO.** When TOTO was used 2 to 8 % of the untreated cells and HP exposed cells were labeled, which indicates that 92 to 98 % of the cells was viable. Even if only 0.24 % of the HP exposed cells were viable according to plate counts, TOTO labeling indicated 92 % viability (data not shown). Furthermore, labeling cells 2.5 hours after exposure to 1.2 mol l^{-1} HP instead of directly after exposure did not lower the viability indicated by TOTO (data not shown). Only TOTO-labeling of cells exposed to the high HP concentrations was significantly different from plate counts. The results may suggest that HP interfered with TOTO labeling. However, labeling was not a problem when cells were first killed by 70 % ethanol, washed, then exposed in the normal way to 1.2 mol l^{-1} HP and incubated with TOTO. However, in this case average fluorescence intensity was 0.7 log unit lower than in cells exposed to 70 % ethanol alone (data not shown), which indicates alterations in the DNA of the cells. **CFSE/pHin** All HP exposed cells were labeled with CFSE even after exposure to the high HP concentration, although the fluorescence intensity was 0.5 log units lower than in untreated cells (data not shown). Only for the HP exposed stationary-phase cells the viability according to the pH_{in}-method was significantly different from viability determined by plate counts.

DISCUSSION

In this study we demonstrate that for viability assessment of disinfectant exposed cells application of flow cytometry in combination with carefully selected fluorescent probes can be a rapid alternative for plate counting.

Effect of growth phase on fluorescent labeling. Our results demonstrate that growth phase influences labeling. First of all, growth phase influenced the susceptibility to disinfectants. Exponential-phase *Listeria monocytogenes* cells were more susceptible to BAC than stationary-phase cells as was shown previously [122]. Stationary-phase cells were more susceptible to HP than exponential-phase cells. Similar observations were done in other studies in which bacteria were exposed to oxidative stress [125, 137]. Because of these differences in susceptibility to disinfectants labeling differs when the same concentration of disinfectant is used. Secondly, we found that for TOTO and CTC labeled cells the fluorescence intensity of some samples was higher in stationary-phase cells than in exponential-phase cells. Still, we could use the same criteria to determine viability for cells from both growth phases. Thirdly, for CTC we found that the percentage of labeling for the untreated cells depended on the growth phase. However, the observed labeling differences will not pose a drawback for using the different probes for cells in different growth phases or heterogeneous populations in the future, because they do not interfere with the way a distinction between viable and non-viable cells is made.

CTC labeling. CTC labeling of untreated cells was poor. This poor labeling may be caused by toxicity of CTC [161]. In addition, poor labeling (62 %) of *L. monocytogenes* biofilm cells with CTC has been found [130]. In contrast, Bovill *et al.* [31] found 100 % labeling in free-living exponential-phase and 90 % labeling in stationary-phase *L. monocytogenes* cells. After exposure to HP, CTC-labeling was low even when a concentration was used that gave no significant reduction in plate counts. This effect was also seen by Huang *et al.* [92] when *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* biofilms were exposed to the oxidative agent monochloramine. CTC-labeling of exponential-phase cells exposed to HP remained at the same level even when viability according to plate counts decreased from 83 % to less than 0.5 %. These results demonstrate that CTC can not be used to quantify *L. monocytogenes* viability after exposure to HP and the probe is not very suitable to be used as an alternative for plate counting for BAC exposed *L. monocytogenes* cells.

TOTO labeling. The percentage of BAC exposed cells that excluded TOTO correlated well with the percentage viability determined by plate counts. Several authors also found exclusion of DNA-binding membrane impermeant probes to correlate well with plate counts for cells killed by membrane active compounds: PI exclusion by nisin exposed *Lactobacillus plantarum* correlated well with plate counts [172]. Correlation with plate counts was also found for TOTO exclusion by several lactic acid bacteria exposed to deconjugated bile salts [38] and SYTOX green exclusion by *S. aureus* exposed to BAC [115].

TOTO exclusion by HP exposed cells did not correlate with plate counts at all. Apparently, HP does not damage the membrane so severely that a large molecule like TOTO (mol. wt. = 1303 g mol^{-1}) can enter the cell. Braux *et al.* [32] found similar results for *E. coli* exposed to the oxidizing agent peracetic acid. In their experiments viability according to plate counts decreased with 3 log units, but PI labeling did not change. Other authors found similar results for other stresses. Bunthof *et al.* [39] found less than 0.3 % viability according to plate counts after exposure of *Lactococcus lactis* to 60°C and no change in PI-labeling. Comas & Vives-Rego [48] found less than 0.2 % viability according to plate counts after exposure of *S. aureus* to formaldehyde and 60°C and no change in PI-labeling. The overall conclusion for TOTO is that it is very suitable to assess BAC killing of cells, but not for assessment of HP efficacy. Some authors suggest that loss of membrane integrity is a good [38] or even the best [140] general indicator for cell death. However, our results show that one should be very careful when to use or not to use DNA-binding membrane impermeant probes.

CFSE/pH_{in} results. From the CFSE labeling results we can conclude that esterase-activity is not a good indicator of viability for *L. monocytogenes* after exposure to BAC and HP. Even when viability was very low according to plate counts, esterase-activity was still present. Bunthof *et al.* [39] had similar results for *Lactococcus lactis* exposed to heat, freezing, bile salts and low pH. Vives-Rego *et al.* [175] found the same results for HP-killed cells. An explanation for this long lasting esterase activity may be that its activity is energy independent [175]. However, the correlation of the pH_{in}-method with plate counts was good for most cells exposed to the low concentrations of disinfectant, largely because of large standard error in the results. For the cells exposed to the high concentrations of disinfectant only the results of the exponential-phase cells exposed to HP correlated well with PC. This is mainly caused by the quantitative method that was used to present the results (compare for an example figs 4.3 and 4.5). In general, the pH_{in}-method results indicated that the ability to maintain a pH-gradient was largely affected by BAC and HP before a major loss in viability according to plate counts took place. The ability to maintain a pH gradient was completely lost only when no viability according to plate counts was found. Thus, the pH_{in}-method is a good indicator for viable cells, for sub-lethal damage and for complete loss of viability in HP and BAC exposed cells, but it is not suitable to quantitatively determine viability in *L. monocytogenes*. Summarizing, for BAC exposed cells TOTO exclusion was the method that correlated best with plate counts. For HP exposed cells the best of the three fluorescence methods was the pH_{in}-method.

ANC state. Our results did not indicate that BAC exposed cells were in an ANC state, because fluorescent labeling gave similar results as plate counts. For HP exposed cells TOTO results could indicate an ANC state. Although HP exposed cells in this study had an intact membrane, most other cell activities were not present anymore; cells were not able to maintain a pH-gradient, did not contain ATP and respiratory activity was low or absent (data not shown). Furthermore, our results indicated that the cells had altered DNA, which will probably have resulted in impairment of cell replication. Thus, even though the cells had an

intact membrane they were not active anymore and very likely unable to replicate and consequently not in an ANC state.

Disinfectant mechanisms of action. In a previous study [122] we found a good correlation between cell death by BAC and complete inhibition of respiration and complete leakage of ATP (mol. wt. = 503 g mol^{-1}) from the cell, which indicates formation of large holes in the cell membrane. The use of fluorescent probes confirmed these results: CTC indicated a complete inhibition of respiratory dehydrogenase activity when plate counts indicated complete loss of viability and labeling by TOTO (mol. wt. = 1303 g mol^{-1}) indicated the formation of large holes in the membrane. The results are in accordance with the mechanism of action of BAC: alteration of the barrier function of the cell membrane leads to leakage of metabolites and coenzymes and disturbance in the delicate balance of metabolite concentrations within the cell [135]. Furthermore, in a previous study we found that the loss of the ability to maintain a pH-gradient in buffer of pH 7.2 did not correlate with cell death. The pH-gradient was dissipated at non-lethal concentrations of BAC [122]. In this study we found only complete loss of the ability to maintain a pH-gradient in a buffer of low pH when plate counts indicated complete loss of viability. Apparently, the capacity to maintain a pH-gradient in a buffer of low pH is correlated with cell viability.

HP oxidizes or forms free radicals [1, 86] which affect enzymes and proteins, DNA, membranes and lipids resulting in damage of transport systems and receptors, difficulty in maintaining ionic gradients, impairment of replication and (in)activation of enzyme systems [1, 86, 158]. CTC results indeed indicate that the enzymes from the respiratory chain [147] were affected. They may have been directly attacked or the cellular membrane may have been disturbed. Notably, TOTO results demonstrated that the damage to the cellular membrane was not so extensive that TOTO could enter the cells. When TOTO was able to enter the HP-exposed cells because of membrane disruption by ethanol (used in the control experiments), TOTO fluorescence in the cells was significantly lower than after exposure to ethanol alone. This may indicate that DNA damage by HP affected binding of TOTO. This assumption is supported by the results of Mortimer *et al.* [138] who found that antimicrobials targeted at nucleic acids influence the degree of staining with DNA binding membrane impermeant probes. pH_{in}-method results showed that cells had difficulty in maintaining a pH gradient after exposure to a low HP concentration and that the complete collapse of the machinery to maintain this gradient coincides with cell death according to plate counts.

General conclusion. Our results demonstrate that even if the mechanism of action of a disinfectant is known, it is still difficult to predict if a certain fluorescent probe is suitable for viability assessment. This is because it is not known which damage is lethal and how extensive the damage to certain cell constituents is. Lisle *et al.* [119] proposed that we should use multiple indices of physiological activity in viability testing. This will provide insight in the overall effect of a disinfectant on physiological activity and on the site(s) of sub-lethal injury [119]. In any case it is very important that a fluorescent probe is tested under the stress conditions it will be used for. Furthermore, when fluorescent probes are used together with

FCM the combination can at this moment not completely replace the official method (plate counting [10]) that is used for viability detection after disinfection because the sensitivity of FCM is not high enough. For disinfectant testing a 5-log reduction in viable cells has to be detected, with FCM the lower limit is currently a 2 to 3-log reduction depending on the signal to noise ratio. FCM can be used as a supplement, for instance for screening strains for possible resistance, testing new disinfectants and studying synergistic effects between disinfectants and additives [115]. Thus, when the proper fluorescent probe is selected, FCM can be used as a rapid alternative for viability assessment by plate counting. Our next step is to investigate if the results from this study can be successfully used for viability assessment of biofilm cells.

ACKNOWLEDGEMENT

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Chapter 5

Development of a standard test to assess the resistance of *Staphylococcus aureus* biofilm cells to disinfectants

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A standardized disinfectant test for *Staphylococcus aureus* cells in biofilms was developed. Two disinfectants, the membrane active benzalkonium chloride (BAC) and the oxidizing agent sodium hypochlorite (NaOCl) were used to evaluate the biofilm test. *S. aureus* formed biofilms on glass, stainless steel and polystyrene in a simple system with constant nutrient flow that mimicked as closely as possible the conditions used in the current standard European disinfectant tests (EN 1040). The biofilm that was formed on glass contained cell clumps and extracellular polysaccharides. The average surface coverage was 60 % and most of the biofilm cells were viable (92 %). Biofilm formation and biofilm disinfection in different experiments were reproducible. For biofilms exposed to BAC and NaOCl a 50 and a 600 times higher concentration was needed, respectively, to achieve 4 log killing than for the cells from the European phase 1 suspension test. Our results show that a standardized disinfectant test for biofilm cells is a useful addition to the current standard tests.

Submitted for publication

INTRODUCTION

Every year foodborne diseases cause millions of illnesses worldwide [49, 134, 171]. One way food can be contaminated with pathogens is through contact with food processing equipment. Therefore, it is of the utmost importance that this equipment is cleaned and disinfected regularly and sufficiently. Thus, an effective disinfectant should be used and an appropriate concentration of this disinfectant should be applied. Too low a concentration increases the risk of food contamination and of resistance acquirement to the disinfectant and too high a concentration will increase the costs and the environmental burden.

The procedure for the testing of candidate disinfectants in Europe consists of 3 phases. In phase 1 the basic activity of the product is tested in a suspension test. Phase 2 consists of two steps. In the first step the product is tested in a suspension test under conditions representative of practical use. The second step consists of other laboratory tests e.g. handwash, handrub and surface tests simulating practical conditions. Phase 3 consists of field tests under practical conditions [8, 9, 10]. Like in Europe, in the USA disinfectants are predominantly tested on cell suspensions [19]. Concerns have been expressed about the European phase 1 and phase 2 step 1 tests for bactericidal testing [114, 125, 148] and suggestions have been made for improvement [114, 125]. But still there are some concerns. A good test must be able to predict the value of the disinfectant in practice [156], and in practice cells can be found much more frequently on surfaces than in suspension. Thus, the question is if suspension test cells are really representative of the cells in practice. In this light in the USA the AOAC hard surface carrier test method is used [16, 17, 18]. In this surface test a suspension of cells is put on a surface and dried for 45 min. Then the disinfectant is applied. In Europe a new surface test is being developed for phase 2 step 2 [29, 107] which uses a similar procedure. These surface tests are already a step forward compared to suspension tests. Still there can be some concern about the suitability of the surface tests. The cells in a surface test only have time to attach to the surface and not to grow, whereas it is known that attached cells that are allowed time to grow form biofilms. Biofilms are much more resistant to antimicrobials than free-living cells [41, 50, 181] and may act as continuous sources of food spoilage bacteria and pathogens that contaminate food, if this increased resistance is not taken into account during disinfection. Therefore, there is a need for a disinfectant test for biofilm cells.

The aim of this study was to develop a standard disinfectant test for biofilm cells. For this study we used *Staphylococcus aureus* DSM 799 because it is used as the representative for Gram-positive bacteria in the USA and European standard tests [9, 10, 19] and it is able to form biofilms [3, 81, 146]. The membrane active compound BAC and the oxidizing agent NaOCl were used, which both are commonly applied in the food industry as disinfectants [149, 186].

MATERIALS AND METHODS

Bacterial strain and growth conditions. *S. aureus* DSM 799, corresponding to ATCC 6538, stock cultures were kept at -80 °C with 25 % wt/vol glycerol added. Cells were grown at 30 °C. Following the prescription of the European phase 1 suspension test [10] cells were grown on tryptone soy agar (TSA) for 24 h and transferred to fresh TSA for another 24 h incubation. Then cells were suspended in peptone physiological salt solution (pps; 1 g of neutralized bacteriological peptone and 8.5 g of NaCl per liter) to an $O.D._{620\,nm}$ corresponding to 1.5×10^8 to 5×10^8 CFU ml^{-1}. This is the phase 1 standard test suspension. Cells that were used for comparison of their diameter to that of biofilm cells were first statically grown overnight in tryptone soy broth (TSB).This culture was then used to inoculate (2 % vol/vol inoculum) 10 ml of ten times diluted TSB (1/10 TSB). For aerobically grown cells this new culture was transferred to a 100 ml Erlenmeyer flask and shaken in a gyrorotatory incubator at 130 rpm. For anaerobically grown cells this culture was transferred in a 60 ml closed vessel and resazurin (1 mg l^{-1}) was added as an indicator for presence of oxygen. Oxygen was depleted after 3 hours. Samples were taken at 2.5, 8 and 24 hours after inoculation.

Chemicals and disinfectants. The disinfectants used in this study were 50 % alkyl-benzyl-dimethylammonium chloride, alkyl distribution from C_8H_{17} to $C_{16}H_{33}$ (BAC; Lamers & Pleuger, Den Bosch, The Netherlands) and sodium hypochlorite with 130 g l^{-1} active chlorine (NaOCl; Acros, Geel, Belgium). For both disinfectants 10 and 100-fold dilutions in demineralized water were prepared from the stock solutions before each experiment and used immediately. TSB, TSA and neutralized bacteriological peptone were from Oxoid (Basingstoke, United Kingdom). Glycerol was from Fluka Chemie AG (Buchs, Switzerland), lecithin from soybeans from BDH laboratory supplies (Poole, England), Congo red from Aldrich Chem. Co. (Milwaukee, USA), sodium lactate was from PURAC biochem. BV (Gorinchem, The Netherlands) and resazurin was from Janssen (Geel, Belgium). TOTO (Quinolinium, 1-1'-[1,3-propanediylbis[(dimethyliminio) -3,1-propanediyl]]bis[4-[(3-methyl-2(3H)-benzothiazolylidene) methyl]]-, tetraiodide) was from Molecular Probes Europe BV (Leiden, the Netherlands). All other chemicals were from Merck KGaA (Darmstadt, Germany).

Biofilm production. Biofilms were grown in a simple set-up (Fig. 5.1) that consisted of a vessel with 1/10 TSB, a pump (Masterflex model no. 7521-10, Cole-Parmer instrument co. Chicago, USA), a culture container (perfusion culture container nr. 4702, Minucells and Minutissue, Bad Abbach, Germany) and a vessel with waste, all connected by silicon tubing. 23 Coupons (13 mm diameter) each held by a coupon carrier (Minucells and Minutissue) placed in the culture container were used as the surface for biofilm formation. For all experiments we used glass coupons (Deckglaeser, Menzel Glaeser, Braunschweig, Germany), except when we mention use of polystyrene (Thermanox plastic coverslips, Nalge Nunc Int., Naperville, USA) or stainless steel (custom made from austenitic stainless steel 304 AISI, werkstofnr. 1.4301, ODS, Barendrecht, The Netherlands).

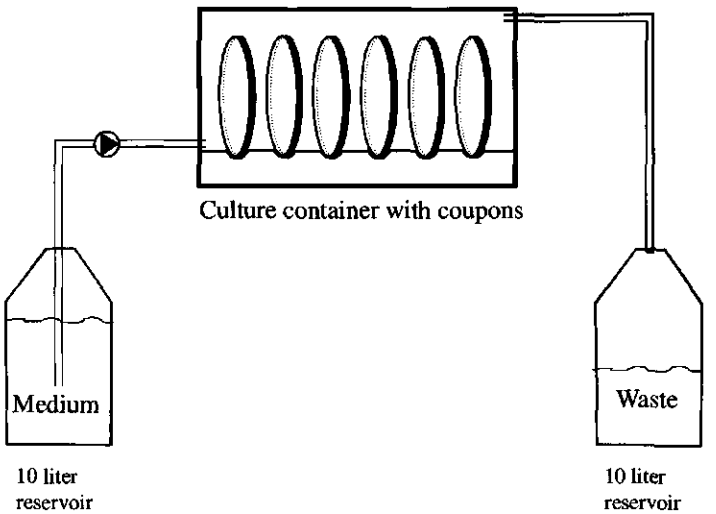

Figure 5.1 Schematic representation of the practical set-up for biofilm formation in the culture container (external measures $l \times w \times h$ = 12.5 cm × 4 cm × 3.5 cm, internal volume ± 22.5 ml). In reality the culture container contains 23 coupons (Ø 13 mm) each held by a coupon carrier.

All coupons and supports were cleaned with 70 % ethanol and autoclaved before use. Before inoculation the coupons were placed in the culture container and 1/10 TSB was pumped (D = 17/h) through the system for 1 hour. The coupons were then inoculated by removing 9 ml of medium from the culture container, pipetting 9 ml of the phase 1 standard test suspension (see above) in it, waiting for 30 min, removing the cell suspension and adding 9 ml of fresh medium. Then the pump was started again and the biofilms were allowed to develop for 24 hours at 30 °C under a constant nutrient flow. Then the pump was stopped, the coupons were removed from the culture container, the supports were removed with tweezers, the coupons were washed by dipping in pps once and then they were ready for analysis or exposure to disinfectants.

Biofilm characteristics. To determine the pH and concentrations of glucose, acetic acid and lactic acid 10 ml samples were taken from the medium vessel and from the tube connecting the culture container and the waste vessel. Both samples were filter (0.2 µm) sterilized and their pH was measured with a PHM240 pH/ion meter from Radiometer (Copenhagen, Denmark). The concentrations of glucose, acetic acid and lactic acid were determined by HPLC of a 20 µl sample and compared with standards. For HPLC we used an Aminex HPX-87H 300 × 7.8 mm column (Biorad, Veenendaal, The Netherlands) and elution at 40 °C with 5 mol l^{-1} H_2SO_4 at 0.6 ml min^{-1}. The eluate was monitored using a refractive index detector.

Photomicrographs of *S. aureus* cells were taken at 400 and 1,000 times magnification with a MC80 camera (Carl Zeiss, Oberkochen, Germany) on an Axioscope phase contrast microscope. Extracellular polysaccharide formation was monitored by incubating biofilms for 40 min with a 0.1 % Congo red (stains polysaccharides [54]) solution and washing two times

in phosphate buffered saline (PBS) (0.2 g of KCl, 0.2 g of KH_2PO_4, 1.5 g of Na_2HPO_4 and 8.0 g of NaCl per liter, adjusted to pH 7.2 with HCl). For the determination of the cell diameter at least 50 cells were measured on photomicrographs and corrected for the difference in magnification between the photomicrographs and the image under the microscope.

To determine the percentage viable cells biofilms were incubated in 9 ml PBS containing 0.3 µmol l^{-1} TOTO for 15 min and examined under an Axioscope microscope equipped with a 50-W mercury arc lamp and a fluorescein isothiocyanate filter set (excitation 450 to 490 nm, emission > 520 nm). To confirm the viability results a biofilm cell suspension was prepared by swabbing the surface and vortexing as described below in 3 ml of pps. The concentration of viable cells was determined by diluting the cell suspension in pps and enumerating by plating on TSA and incubating for 48 h at 30 °C. The total concentration of cells was determined by counting the suspension under a phase contrast microscope using a Bürker-Türk counting chamber, with a depth of 0.01 mm at a 1,000 times magnification. For each sample 63 squares of 0.0025 mm^2 were counted. The percentage of viable cells was calculated by dividing the concentration of viable cells by the total concentration of cells and multiplying by 100 %. Experiments were performed in quadruplicate. The percentage surface coverage was calculated by dividing the average biofilm formation (in CFU cm^{-2}) by the percentage of viable cells and multiplying this number by the area covered by one biofilm cell (πr^2, r = 0.5 × average diameter). EPS was excluded from the calculation.

Killing experiments. All killing experiments were done at 20 °C. Biofilms were grown and washed as described above. One coupon was added to 3 ml of disinfectant or for the control to 3 ml of demineralized water in a closed 50 ml tube. 3 ml was chosen to achieve approximately the same cell concentration per ml of disinfectant as in the phase 1 test. After 5 min 27 ml of neutralizer was added, which consisted of 10 ml of a 34 g l^{-1} KH_2PO_4 buffer adjusted to pH 7.2 with NaOH, 3 g of lecithin from soy beans, 30 ml of Tween 80, 5 g of $Na_2S_2O_3$ and 1 g of L-histidine per liter. After another 5 min the coupon that was still in the liquid was swabbed (polyester fiber-tipped applicator swab, Becton Dickinson and Company, Sparks, USA) twice on both sides and the tube containing the swab and the coupon was vortexed at full speed for 30 s to remove all biofilm cells from the swab and the surface. We tried several other methods to remove the biofilm cells from the coupons: shaking or vortexing with glass beads, vortexing, and sonication. The swab-vortex method gave the best removal from the surface and the highest number of CFU cm^{-2} (results not shown). An appropriate dilution of the neutralized suspension was made in pps and the sample was enumerated by spiral plating on TSA immediately after dilution. In the original suspension test pour plates are used, but Langsrud and Sundheim [114] showed that the use of pour plates reduced the number of surviving *S. aureus* cells exposed to BAC significantly. The plates were incubated at 30 °C and the colonies were counted after 48 h. Killing experiments were done in quadruplicate and in a way that prevented bias in the results due to the place of the coupon in the culture container. This place might influence the amount of biofilm formed

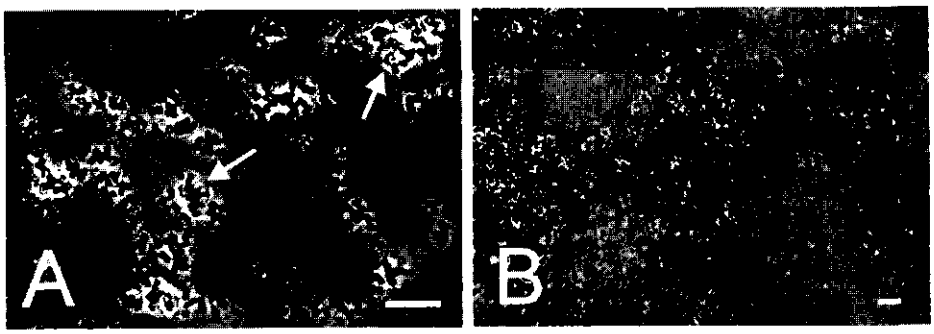

Figure 5.2 Light microscopic image of a 24 h *S. aureus* biofilm on glass after washing in pps. Images were taken at a magnification of 1000 times (A) and 400 times (B). Bar is 10 μm. The bright material accompanying the clumps of cells represents extracellular polymeric substances (indicated by arrows).

on the coupon or the physiological status of the cells. Therefore, the 20 coupons used for one killing experiment were divided in four groups (coupon 1-5, coupon 6-10 etc.). For each of the 5 treatments a coupon was taken at random from each of the four groups. For the killing of suspended biofilm cells, cells were first removed from the surface into 3 ml of demineralized water as described above and consecutively a small volume of concentrated disinfectant or demineralized water for the control was added. After 5 min 27 ml of neutralizer was added (see above). Further analysis was done as for biofilm cells. Phase 1 test cells were grown and killed according to the phase 1 test as described earlier [125]. All data were statistically analyzed with a paired students t-test with two tailed distribution and a 0.05 confidence level. The null hypothesis was that there was no significant difference between the viability of the different cell types.

RESULTS

In this study we developed a standard test to assess the efficacy of candidate disinfectants against *S. aureus* growing as a biofilm on glass. Besides glass (negatively charged) polystyrene (hydrophobic) and stainless steel (negatively charged) also proved to support the growth of biofilms. Biofilm formation, as determined by plate counts, did not differ significantly on the three types of surfaces (data not shown). We used glass because it is negatively charged, like stainless steel that is used abundantly in food industry. Furthermore, glass was used because it is easy to observe formation, removal and staining of biofilms on this surface with phase contrast microscopy.

Cells growing as biofilms were characterized microscopically (Fig. 5.2). At a high magnification we observed single cells, diplococci and big clumps of cells attached to the surface (Fig. 5.2A). The cells in the clumps were surrounded by yellowish material that represents extracellular polymeric substances. This was confirmed by staining the biofilm

Table 5.1 Cell diameter of *S. aureus* cells cultured under a variety of conditions.

Cell growth	Medium	Aerobic/anaerobic	Time (h)	Cell diameter in μm[a]
Planktonic	1/10 TSB	Aerobic	2.5	1.12 ± 0.12
Planktonic	1/10 TSB	Aerobic	8	0.97 ± 0.13
Planktonic	1/10 TSB	Aerobic	24	0.86 ± 0.12
Agar (phase 1 test cells)	TSA	Aerobic	24	1.08 ± 0.19
Planktonic	1/10 TSB	Anaerobic	2.5	1.11 ± 0.13
Planktonic	1/10 TSB	Anaerobic	8	1.04 ± 0.18
Planktonic	1/10 TSB	Anaerobic	24	Cell lysis
Biofilm	1/10 TSB	Unknown	24	0.93 ± 0.24

[a] Values are an average of at least 50 cells.

Table 5.2 Characteristic parameters of influent and effluent of the culture container after 24 h continuous feeding with 1/10 TSB.

Parameter	Influent[a]	Effluent[a]
Glucose concentration (in mmol l^{-1})	1.2	0.29
Acetic acid concentration (in mmol l^{-1})	0	1.27
Lactic acid concentration (in mmol l^{-1})	0	0.87
pH	7.5	6.6

[a] Values are an average of 6 experiments

with the polysaccharide stain Congo red (results not shown). A lower magnification showed that the biofilm fully covered the glass surface over a wide area (Fig. 5.2B). This observation was confirmed by the calculated 60 % surface coverage. This percentage was calculated with help of the average biofilm formation of $8.1\times10^7 \pm 4.4\times10^7$ CFU cm^{-2}, the percentage viable cells in the biofilm of 92 ± 27 % and the average diameter of the biofilm cells (Table 5.1). To further characterize the biofilm cells their diameter was compared to that of cells grown under various other conditions (Table 5.1). The longer the planktonic cells were grown, the smaller they became. This was true for anaerobically and aerobically grown cells. The diameter of biofilm cells was most comparable to that of cells that were grown aerobically between 8 and 24 hours in 1/10 TSB. Analysis of inflowing medium (influent) and outflowing waste (effluent) gave an indication of the nutrient supply and nutrient consumption in the biofilms (Table 5.2). At 24 h almost all glucose was being consumed and converted to the end products of *S. aureus* aerobic (acetic acid) and anaerobic (lactic acid) glucose metabolism, in the proportion 10 to 7. Acid formation was confirmed by the pH drop of about one unit.

Fig. 5.3 shows that the biofilm formed using the new method is reproducible. Biofilm

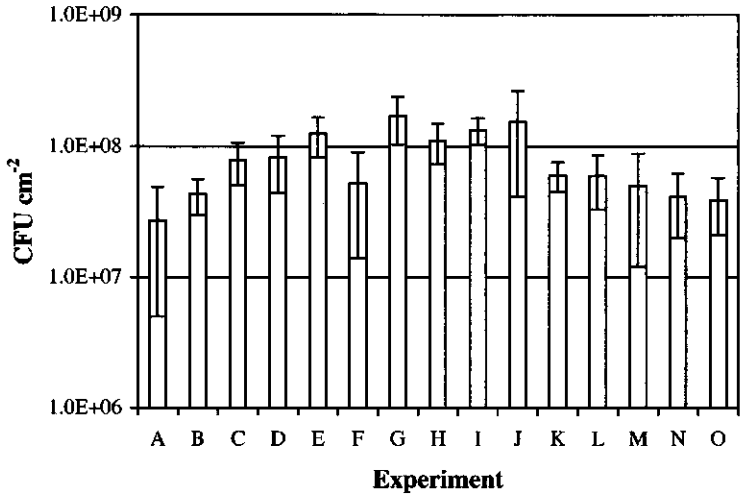

Figure 5.3 Average biofilm formation per experiment after 24 h by *S. aureus* in fifteen independent experiments. Error bars show the standard deviation. Values are an average of at least 4 coupons.

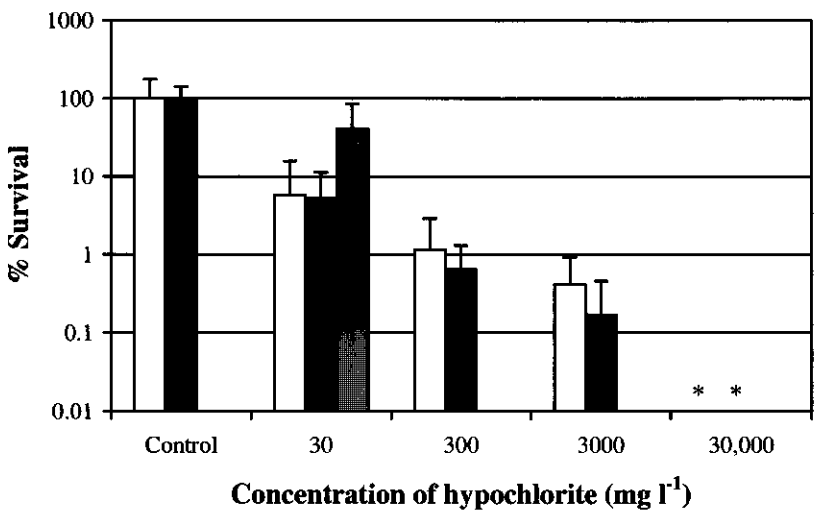

Figure 5.4 Survival of *S. aureus* biofilm cells after 5 min exposure to 0, 30, 300, 3000 and 30,000 mg l^{-1} of sodium hypochlorite in two separate experiments (white and black bars) and survival of *S. aureus* phase 1 test suspension cells exposed to 30 mg l^{-1} of sodium hypochlorite (gray bar, separate result, taken from [125]). Error bars show the standard deviation. Values are an average of at least 4 coupons. * % Survival is below detection limit of 0.01 %

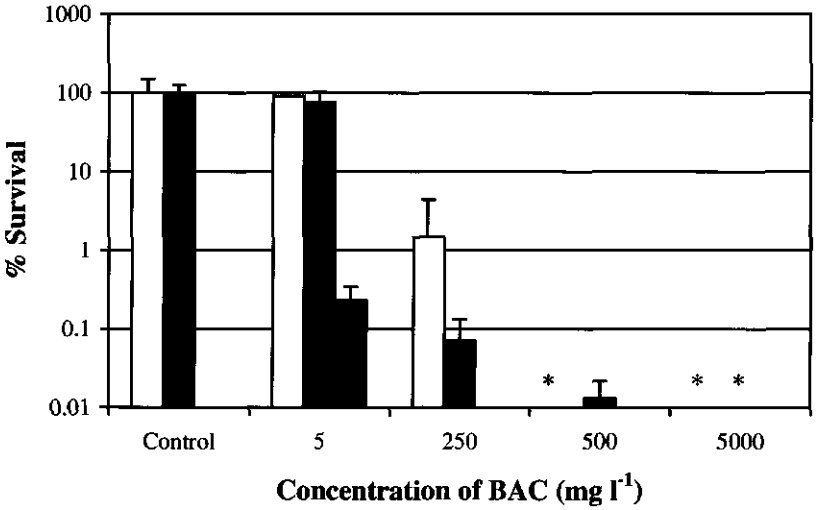

Figure 5.5 Survival of *S. aureus* biofilm cells after 5 min exposure to 0, 5, 250, 500 and 5000 mg l^{-1} of BAC in two separate experiments (white and black bars) and survival of *S. aureus* phase 1 test suspension cells exposed to 5 mg l^{-1} of BAC (gray bar, separate result, taken from [125]). Error bars show the standard deviation. Values are an average of at least 4 coupons. * % Survival is below detection limit of 0.01 %

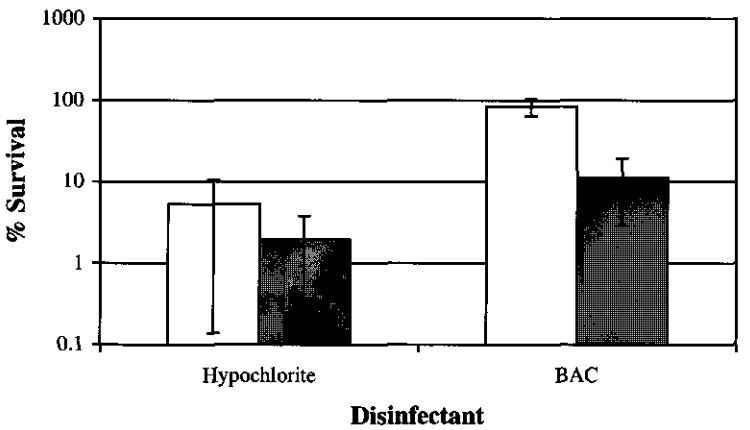

Figure 5.6 Survival of S. *aureus* biofilm cells (white bars) and suspended biofilm cells (grey bars) after 5 min exposure to 30 mg l^{-1} of sodium hypochlorite (A) or 5 mg l^{-1} BAC (B). Error bars show the standard deviation. The experiment was performed in quadruplicate

formation in these 15 independent experiments was between 4×10^7 and 1.3×10^8 CFU cm^{-2}. The error bars in Fig. 5.3 demonstrate that the biofilm formation on the different coupons in one experiment varied. To prevent errors in the results of the disinfectant testing due to these or other variations in biofilm formation we used a solid statistical method for coupon sampling during disinfectant testing (see Material and Methods).

The results of the disinfectant tests are shown in figs 5.4 and 5.5. Both these figures represent the results of two separate biofilm experiments and a separate planktonic cell experiment. Survival of biofilm cells and phase 1 test cells exposed to 30 mg l^{-1} of hypochlorite did not differ significantly (1.2-log, 1.3-log and 0.38-log reduction, Fig. 5.4). To achieve more than 4-log reduction a concentration of 30,000 mg l^{-1} of hypochlorite was needed. For phase 1 cells only 50 mg l^{-1} was needed to achieve more than 5-log reduction (data not shown). 5 mg l^{-1} of BAC had almost no effect on the viability of biofilm cells (0.041-log and 0.12-log reduction), whereas phase 1 test cells showed 2.6-log reduction when exposed to the same concentration of BAC (Fig. 5.5). To achieve at least a similar reduction of 2.6 log for biofilm cells a BAC concentration of 250 mg l^{-1} was needed. A concentration of 500 mg l^{-1} gave almost a 4 log unit reduction. For phase 1 cells only 10 mg l^{-1} was needed to achieve a 5-log reduction (data not shown).

To find out whether or not the attachment to the surface contributes to resistance biofilm cell and suspended biofilm cell survival was determined (Fig. 5.6) Biofilm cell survival after exposure to hypochlorite did not differ from that of suspended biofilm cells. For biofilm cells exposed to BAC survival was about 8 times higher (0.88 log units difference) than for suspended biofilm cells.

DISCUSSION

In this study we describe a standard test to assess the activity of candidate disinfectants against *S. aureus* biofilm cells. Such a standard test has to meet several requirements. In the first place a disinfectant test should be as simple as possible and not require specialized or expensive pieces of laboratory equipment. Furthermore, the test should be repeatable and reproducible [90]. Finally, for convenience it is advisable to stay as close as possible to the tests that have already been approved. Our biofilm test meets all these requirements. Our biofilm forming system is very simple, relatively cheap, the test can be performed in one week and all equipment and material is commercially available. Furthermore, the test is as similar as possible to the phase 1 suspension test and the test is reproducible and repeatable within the limits of the phase 1 test. The phase 1 test allows the concentration of cells to be tested to vary between 1.5×10^8 and 5×10^8 CFU ml^{-1}. Thus, a 3.3 times difference in cell concentration is allowed. Our results are within this limit. Jackson *et al.* [95] found higher variation (2×10^6 -1×10^8 CFU cm^{-2}) when they tried to grow reproducible biofilms of *Pseudomonas* and *Klebsiella* in a flowing system. This shows that it is very difficult to stay

within the limit that is set by the phase 1 test. The reproducibility of disinfection in our test was the same as what we found for the phase 1 suspension test [10].

The specific requirements that a biofilm test has to meet are that a genuine biofilm is produced with the method that is used. Thus, cells have to attach to and grow on a surface and the cells have to form extracellular polymeric substances [178]. Furthermore, to obtain firmly attached cells biofilm formation is best in a system in which shear stress is constantly applied on the biofilm cells, for instance by flow of the medium along the surface for biofilm formation. These specific requirements for a biofilm test are also met in our test. For biofilm formation we use a system with constant shear stress. In this system *S. aureus* formed a genuine biofilm after 24 hours as clumps of cells with extracellular material could be observed. The number of cells on the surface was quite high. Other authors found *S. aureus* biofilm formation between 5×10^6 and 8×10^7 CFU cm^{-2} after 24 to 48 h in batch systems in rich media at temperatures from 35 to 37 °C [3, 81, 146]. Further characteristics of the biofilm cells were that after 24 h they did not have enough oxygen to grow completely aerobic anymore and were not glucose limited. The size of the biofilm cells was quite large (0.93 µm). Several authors found smaller sizes (0.5-0.7 µm) for *S. aureus* biofilm cells of 12-24 h [146, 184, 192]. This might be caused by growth of these biofilm cells in a batch system where nutrient limitation and the accumulation of waste starts much earlier than in a continuous flow system. The size of planktonic cells appeared to be related to growth rate as was previously also described by Williams *et al.* [184]. These authors concluded that this was also true for biofilm cells. We found that biofilm cell size was most similar to that of aerobically grown cells in late logarithmic phase. The large variation in size indicated variety in growth phase in the biofilm cells.

All requirements for a biofilm disinfectant test are fulfilled in our test. The criterion for a candidate disinfectant to pass the test are that there should be more than a 4-log reduction in viability in 5 minutes in a biofilm that contains in between 4×10^7 and 1.3×10^8 CFU cm^{-2}. Biofilm test cells appear to be much less susceptible to disinfectants than phase 1 test cells, especially at the concentration needed to reduce the viability of the cells more than 4 log units. Furthermore, our results show that increased resistance of biofilm cells is only partly caused by being attached to a surface. Other factors that may be responsible for the increased resistance of the biofilm cells are the presence of extracellular polymeric substances, the different physiology of the biofilm cells due to attachment and the variation of physiologies of the biofilm cells due to variation in growth phase and oxygen concentration.

It was already known that *S. aureus* biofilm cells are more resistant to antibiotics than free-living cells [3, 14, 185]. Oie *et al.* [146] showed that that this is also true for disinfectants. They exposed 24 h biofilms of methicillin-resistant *S. aureus* grown in TSB on silicone disks in a batch system to several disinfectants. To obtain more than a 4-log reduction of biofilm cell viability in 10 minutes 10,000 mg l^{-1} of BAC and 1,000 mg l^{-1} of hypochlorite was needed. Some preliminary results of the surface test that is being developed

for phase 2 step 2 [107] show that in this test about 100 times more BAC is needed to achieve the same kill for surface test cells as for phase 1 test cells, which is comparable to our results. However, for hypochlorite only 20 times more disinfectant is needed to achieve the same kill, whereas for our biofilm test cells we needed 600 times more hypochlorite. Thus, a standard biofilm test is a useful addition to the current standard tests. We propose that the test could replace the phase 2 step 1 suspension test or be performed as a replacement for or addition to the phase 2 step 2 tests.

Summarizing, we have developed a standard biofilm test that confirms that biofilm cells are less susceptible to disinfectants than suspension test cells. The test may be easily used with other bacteria, such as *Pseudomonas aeruginosa*. The test provides a useful tool to characterize the efficiency of novel and existing disinfectants against biofilms.

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Chapter 6

General discussion

Foodborne bacteria cause millions of food poisonings worldwide every year. One way to prevent (re)contamination of food is adequate cleaning and disinfection of food processing equipment. Disinfectants play an important role in this process. Before a candidate disinfectant can be approved and used as a disinfectant it first has to pass a number of tests. In Europe disinfectants are tested on bacterial cell suspensions in so-called suspension tests. However, bacteria on food processing equipment do not grow in suspension. They are attached to surfaces and form biofilms, a mode of growth that makes them less susceptible to stress conditions, such as exposure to disinfectants. Thus, the current disinfection test procedure would profit from the testing of biofilm cells instead of suspended cells. Furthermore, the disinfectant tests that are currently used employ plate counting for viability assessment of the survivors. This is a time-consuming method (2 days) and rapid alternatives would further improve the method. One such alternative is the use of fluorescent probes, which allow reduction of the time for viability assessment to approximately two hours. Besides these subjects, this chapter discusses several factors that influence the efficacy of disinfection, further developments of disinfectant tests and biofilm control in practice.

FACTORS THAT INFLUENCE THE EFFICACY OF DISINFECTION

In this thesis several factors that may influence the efficacy of disinfectants were studied. Exposure of *Listeria monocytogenes* cells in the presence of glucose or BHI-medium does not influence the efficacy of benzalkonium chloride (BAC) (Chapter 2) and hydrogen peroxide (HP) (data not shown) as compared to exposure in buffer. Exposure of *L. monocytogenes* cells in the absence of oxygen did not influence the efficacy of BAC (Chapter 2) as compared to exposure in the presence of oxygen. It is important to know if oxygen and nutrients have an additional influence on killing, because in biofilms the distribution of these compounds is not even and not constant.

We found that presence of dead cells influences the efficacy of disinfectants (Chapter 3). Dead cells may react with disinfectants either directly for example by binding membrane active compounds such as BAC or indirectly by the contribution of their enzymes, such as catalase in the degradation of HP. In this way they may protect the viable cells in the suspension. Our results show that decline-phase cells indeed are more resistant to disinfectants in the presence of these dead cells, which account for more than 90 % of the *Staphylococcus aureus* decline-phase population.

Another factor that may influence the efficacy of disinfection is growth phase (chapters 2, 3, 4). *S. aureus* exponential-phase, stationary-phase and decline-phase cells were exposed to BAC, HP, dodecylbenzyl sulphonic acid (DSA) and sodium hypochlorite (NaOCl) (Chapter 3). It is assumed that cells in stationary-phase and decline-phase are less susceptible to disinfectants than exponential-phase cells because cells in stationary-phase and decline-phase lack certain nutrients. Bacteria are known to respond to this starvation stress by growth rate reduction and induction of defense mechanisms [35, 57, 61]. As a result, they may become more resistant to other types of stress, such as that caused by disinfectants [55, 59, 72, 87, 109]. In our results, this was the case for *S. aureus* decline-phase cells, but stationary phase cells were less resistant than exponential-phase cells. Watson *et al.* [179] found similar results. They explain the sensitivity of stationary-phase cells with the biphasic death curves they observed for stationary-phase cells. They proposed that the stationary-phase cells that show reduced resistance to the treatments, are the same cells that will die upon long-term starvation. This proposal is based on the concept that the cells become committed to survival or death early after entry into the stationary-phase unless they are rescued by provision of nutrients. In our experiments biphasic death patterns were also observed for *S. aureus* stationary-phase cells exposed to NaOCl and BAC. When exposed to the other disinfectants, *S. aureus* stationary-phase cells died too rapidly to for any conclusions to be drawn.

Similar experiments for *L. monocytogenes* exponential-phase cells and stationary-phase cells exposed to BAC (chapter 2 and 4), HP (Chapter 4), DSA and NaOCl (data not shown) revealed that *L. monocytogenes* stationary-phase cells are less susceptible to surfactants than exponential-phase cells, but more susceptible to oxidizing agents than exponential-phase cells. Decreased susceptibility of *L. monocytogenes* stationary-phase cells to environmental stresses may be explained by induction of the alternative σ-factor, σ^B, in stationary-phase cells. σ^B is involved in regulation of response to environmental stresses in *L. monocytogenes* [23, 70, 182]. This factor is also involved in protection of *L. monocytogenes* stationary-phase cells to oxidative stress [70]. Since σ^B is not induced in unstressed exponential-phase cells it appears that in these cells other mechanisms are involved in protection from oxidative stress.

A difference in catalase activity could be an important factor in the survival of cells exposed to HP. However, *S. aureus* exponential-phase, stationary-phase and solid medium cells showed no substantial differences in catalase activity, although exponential-phase cells die more rapidly than the other two cell types (Chapter 3). Apparently, *S. aureus* exponential-phase cells have a lower inherent resistance to HP than the other two cell types. In a decline-phase cell suspension that contained the same total number of cells as the above mentioned other cell type suspensions the catalase broke down the HP at least 2.5 times as slow as it did in the other cell types. Nevertheless, cell survival was still higher than that of the other cell types. *S. aureus* decline-phase cells apparently have a higher inherent resistance to HP than other cell types. Similar experiments for *L. monocytogenes* exponential-phase and stationary-

phase cells revealed that catalase activity was not substantially different (data not shown). Thus, the difference in susceptibility to HP between these cell types can not be explained by differences in catalase activity.

Growth in a biofilm may influence the efficacy of disinfectants. This may be caused by several factors. First of all, the extracellular material of the biofilm may exclude or influence the access of the disinfectant and the outer layers of the biofilm may react with and quench the disinfectant [37, 112]. Our results showed that susceptibility to NaOCl treatment did not differ significantly in biofilm cells and suspended biofilm cells (Chapter 5). Susceptibility to BAC treatment differed significantly in biofilm cells and suspended biofilm cells, but only by one log unit (Chapter 5). Thus, for the *S. aureus* biofilms used in this study the biofilm matrix is of small importance to disinfectant susceptibility. Additionally, susceptibility of cells in a biofilm may be altered because a cell that is attached to a surface has a different phenotype than a cell grown in suspension. This may be because of the attachment itself, nutrient stress or presence of a large number of cells on the surface (quorum sensing). For the importance of each of these three factors evidence has been found with the help of mutants and reporter genes [56, 62, 180]. Furthermore, our results indicated that cells in biofilms formed by a single strain can differ even genotypically. A 6-day-old biofilm of *S. aureus* DSM 799 grown at 25 °C contained several white (unpigmented) mutants of which two were selected. These mutants were stable and showed increased slime production after 2 days of (planktonic) incubation in TSB (data not shown). It is very likely that all these factors together cause a large heterogeneity in structure and phenotypes in the biofilm, which increases the probability of survival of a small population of cells in the biofilm after exposure to disinfectants. In our results this can be demonstrated by comparing the death curves of free-living cells and then especially exponential-phase cells to those of biofilm cells (Chapter 2, Fig. 2.1 and Chapter 5). Disinfectants have only a very small effect on the viability of free living cells at low concentrations, but after a very small increase of disinfectant concentration a very sharp decline in viability occurs. Biofilm cells show the same response to disinfectants as free-living cells at low concentrations, but a very large increase in disinfectant concentration is needed to cause a substantial decline in viability of biofilm cells.

DISINFECTANT TESTS

There have been concerns about the suitability of the standard European disinfectant tests. Some of the concerns about these suspension tests are the lack of test reproducibility and the predictive value of laboratory grown cultures for naturally occurring strains [114, 148]. Langsrud and Sundheim [114] showed that pre-growth in the absence of oxygen and using spread plates instead of pour plates increases the survival of *S. aureus* after exposure to benzalkonium chloride and grapefruit extract. On the other hand Payne *et al.* [148] showed

that clinical isolates were not more resistant than *S. aureus* DSM 799, the strain that is used in suspension tests.

Apart from the nature of the strain, growth phase could influence the resistance of bacteria. We compared the susceptibility of cells grown according to the European suspension tests [9, 10], on solid medium, with cells in different growth phases grown in liquid medium (Chapter 3). Decline-phase cells were generally the most resistant cell type. Exponential-phase cells were less resistant than decline-phase cells and, surprisingly, stationary-phase cells were the least resistant of the three. Cells grown according to the suspension tests were in none of these tests the most resistant cells. Their survival was 1 to 3 log units less than that of the most resistant cells. This shows that the solid medium cells currently used are not the most resistant type of cells that can be used in a disinfectant test and thus the current suspension tests underestimate the phenotypic resistance of *S. aureus* cells to disinfectants. To improve the test it would be advisable to use cells for suspension tests that are grown differently as compared to the currently used solid medium cells, as was also suggested for *Pseudomonas aeruginosa* [77].

However, we are left with some concerns. A good test must be able to predict the value of the disinfectant in practice [156], and in practice cells can be found much more frequently on surfaces than in suspension. In this light a new European surface test is being developed for phase 2 step 2 [29, 107]. In this surface test a suspension of cells is put on a surface and allowed to dry for 60 min, after which the disinfectant is applied. This test is already a step forward compared to suspension tests. Still there can be some concern about the suitability of the surface test. The cells in a surface test only have time to attach to the surface and not to grow, whereas it is known that attached cells that are allowed time to grow form biofilms and cells in biofilms are generally more resistant to antimicrobials than free-living cells and surface dried attached cells [29, 41, 50, 181]. Therefore, we developed a disinfectant test for biofilm cells. This test may not only help to better predict the efficacy of the disinfectant in practice but may also help to find new or to select existing disinfectants that are particularly effective against biofilms, since up till now candidate disinfectants are not tested on biofilm cells.

In the developed *S. aureus* biofilm test, cells were less susceptible to disinfectants than suspension test cells and, furthermore, one disinfectant (BAC) was relatively more effective against biofilm cells than the other (NaOCl, Chapter 5). This difference may be explained by the fact that BAC can more easily penetrate the biofilm than NaOCl because of its surfactant properties. In addition biofilm cells may be better adapted to oxidative damage than to membrane damaging stresses.

The development of the biofilm test consisted of several stages. First the experimental conditions were selected, such as temperature, medium, strain, inoculum and a reactor for biofilm formation. In this case the reactor was a flow through system with a culture container that can contain 23 coupons on which biofilms are formed (Fig. 5.1). For convenience and to make comparison to previous results easier, the chosen conditions were as close as possible

to the current tests. Furthermore the test was as simple as possible. Ten times diluted TSB was used to prevent too much planktonic growth in the system and because it is generally thought that relatively poor media support biofilm formation [15, 37, 62, 84]. Secondly, the best method to quantify biofilm formation was selected, in this case swabbing the surface of the coupons and vortexing the swab together with the coupon. Thirdly, we assessed the reproducibility of biofilm formation which was in between the limits set by the current suspension test in which a 3.3 times difference in cell concentration is allowed. In the context of standardized biofilm formation Jackson *et al.* [95] found a 50 times difference in biofilm cell concentration for mixed biofilms of *Pseudomonas fluorescens*, *P. aeruginosa* and *Klebsiella pneumoniae*. Ceri *et al.* [42] found approximately a 3 times difference in biofilm cell concentrations for *P. aeruginosa*. Finally, we assessed the reproducibility of disinfection, which was sufficient. Zelver *et al.* found a repeatability standard deviation of 0.66 for *P. aeruginosa* biofilms exposed to chlorine [191].

The following criterion was set for a candidate disinfectant to pass the test: more than a 4-log reduction in 5 min in a biofilm with a cell concentration that falls within the 3.3 times variation allowed in the current suspension tests (Chapter 5). Other authors [186] have proposed that for a biofilm test only a 3-log reduction is necessary, but that is too small a reduction for these biofilms that can contain op to 1.3×10^{8} CFU cm^{-2}.

This biofilm test is a general test that could be used as a replacement for the phase 1 test if a candidate disinfectant will only be used on biofilms, or as an additional test for efficacy testing of disinfectants against biofilms in phase 2 step 1 or 2. For phase 2 step 2 more specific tests could be developed and added such as the ones that already exist for the human mouth, toilet bowls, oilfield water injection systems and cooling water or water distribution systems [63, 150]. Furthermore, these tests should also include the effect of hard water, soil, sanitizers and mechanical action, in order to mimic practical conditions.

Several suggestions can be done to further develop the biofilm test described in this thesis. A number of studies with *L. monocytogenes*, *P. aeruginosa* and *K. pneumoniae* showed that older biofilms (1 to 2 weeks) are more resistant to antimicrobials than young biofilms (1 to 2 days) [14, 15, 74, 116]. However, older biofilms are more difficult to remove from the surface [43] and extension of the current protocol by more than one day would mean that the test can not be performed in one working week anymore. Furthermore, older biofilms may not be very stable because of cycles in biofilm formation as were reported for *L. monocytogenes* biofilms [43] and mixed biofilms of *P. aeruginosa*, *K. pneumoniae* and *P. fluorescens* [95] or because of selection of mutants. Finally, the most important argument is that a biofilm over 24 h old is not very likely to be present on a food contact surface, since in most factories these surfaces are cleaned and disinfected every day [25].

Another suggestion for development of the test is to use another *S. aureus* strain than *S. aureus* DSM 799. Fig. 6.1A shows that several other strains of *S. aureus* are stronger biofilm formers than the currently used *S. aureus* strain. Thus, with the use of one of these strains a thicker biofilm may be developed, which will widen the detection range of the test.

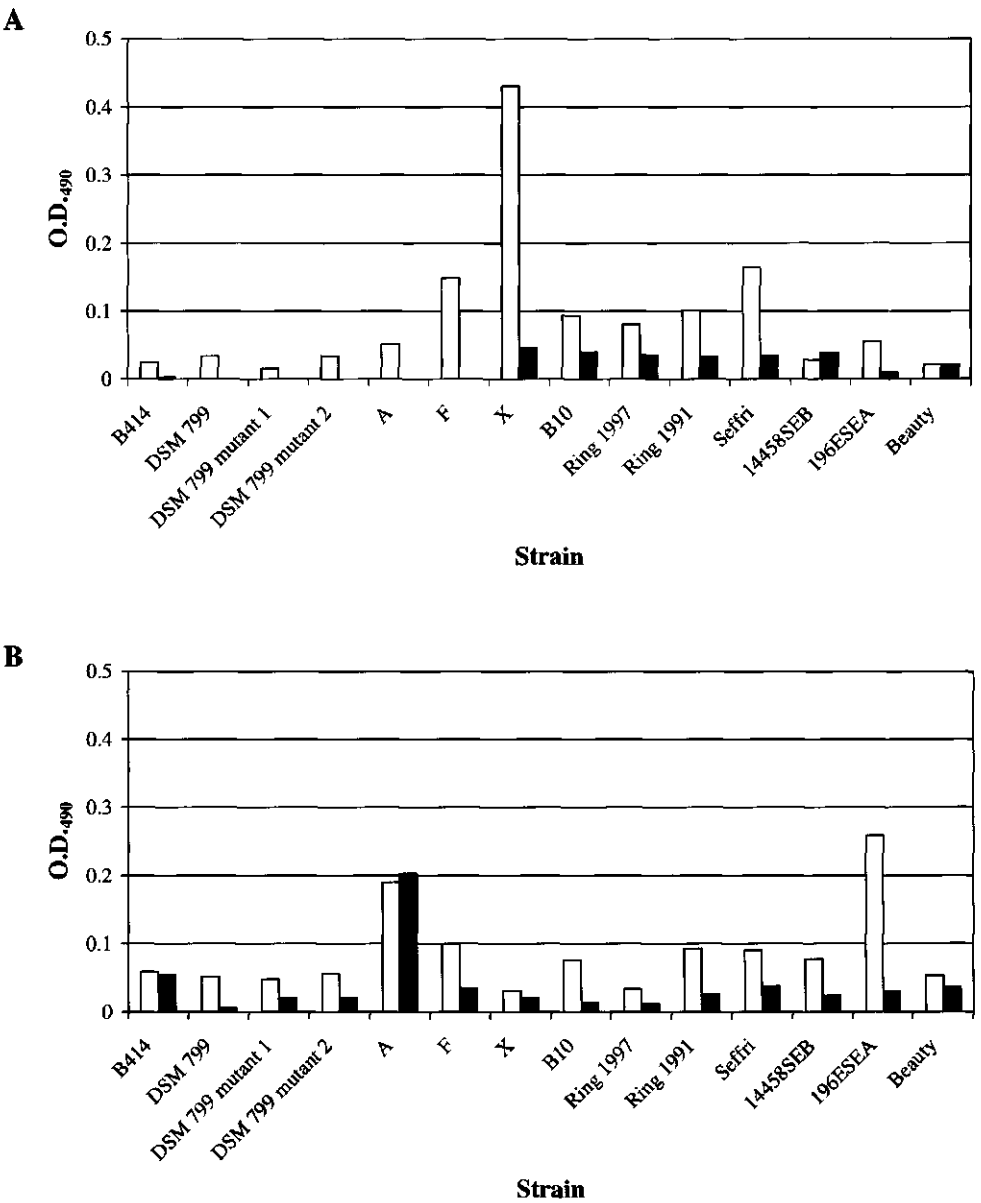

Figure 6.1 Biofilm formation by *S. aureus* strains at 25 °C (A) and 15 °C (B) in a 96-well polystyrene microtiter plate. Wells were inoculated with 200 µl of a 100 times in TSB diluted preculture (grown for 22 h, shaking, at 37 °C, in TSB). After 48 (25 °C) or 194 h (15 °C) of incubation wells were washed twice with PBS [122], fixed with Bouin's fixative solution, stained with saffranine (5 g l^{-1}, total stain, white bars) and Congo red (1 g l^{-1}, polysaccharide stain, black bars), washed twice with PBS, after which $O.D._{490}$ was measured. Values represent the average of 8 wells. Experiment done by Lisette Elsinga.

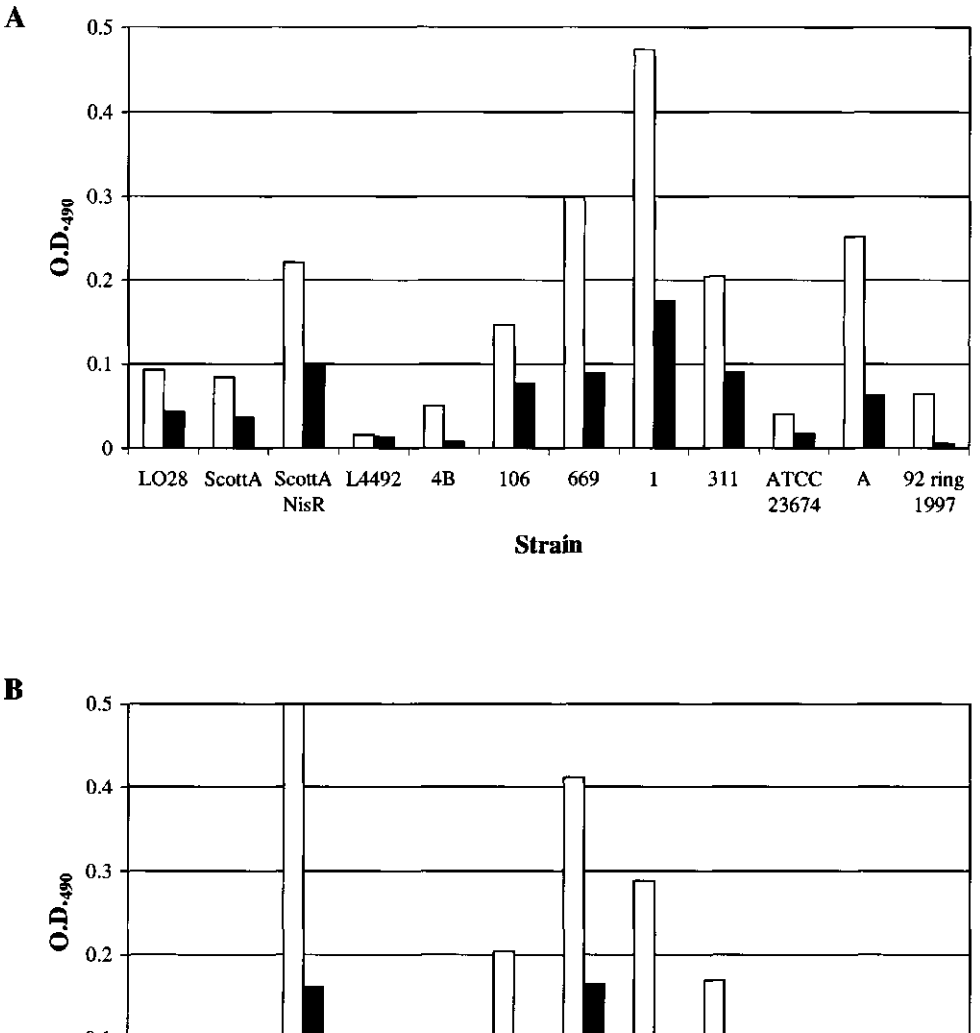

Figure 6.2 Biofilm formation by *L. monocytogenes* strains at 25 °C after 30 h (A) and 15 °C after 148 h (B) according to the method described in Fig. 6.1 (except for the preculture which was grown for 15.5 h, at 30 °C, in BHI and diluted in BHI). Experiment done by Lisette Elsinga

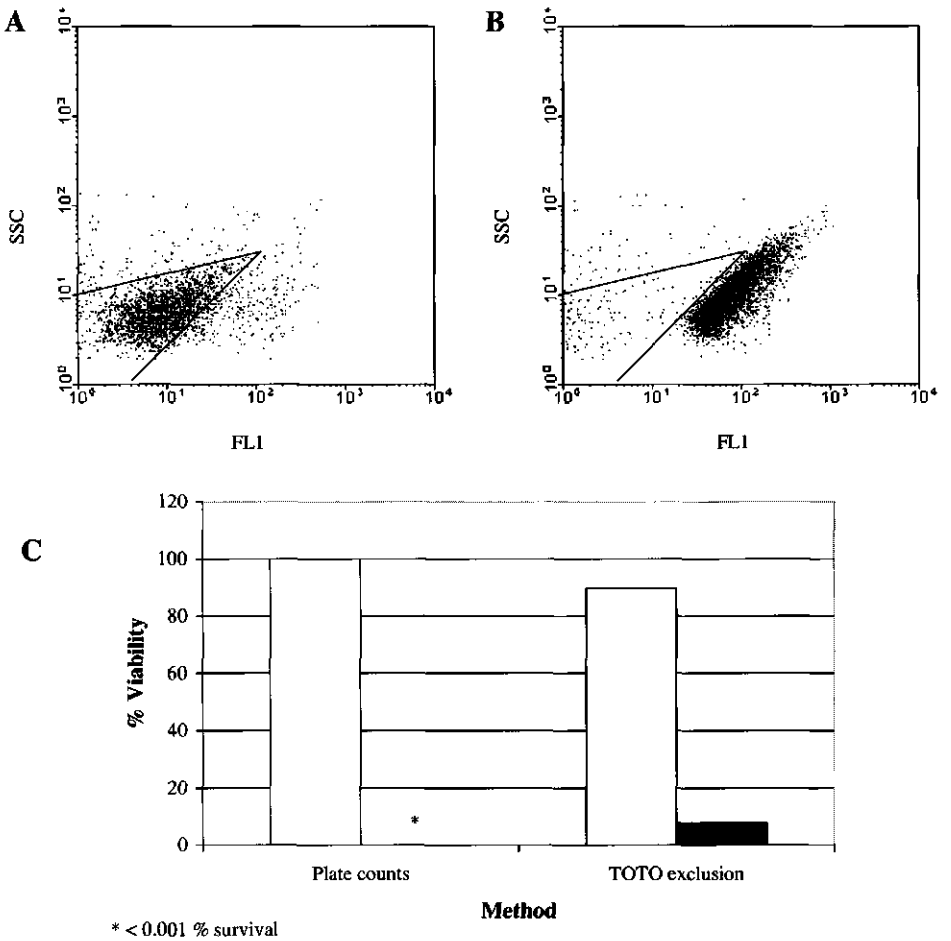

Figure 6.3 Viability assessment of 24-h *S. aureus* biofilm cells (see Chapter 5). Intact biofilms were exposed to 3 ml of water (untreated) or 300 mg l^{-1} of BAC. After 5 min 27 ml of PBS [122] was added. After 5 min cells were removed from the surface (see Chapter 5) and either labeled with TOTO (0.3 µmol l^{-1}, 15 min) without washing and analyzed with flow cytometry or the cell suspension was diluted in PBS, plated on TSA and incubated at 30 °C for 48 h. Flow cytometry results show FL1-fluorescence and SSC of untreated cells (A) and cells exposed to BAC (B) stained with TOTO (the triangle shows the region with unlabeled/viable cells). Furthermore, the percentage viability determined with plate counting and TOTO exclusion is shown (C) for untreated cells (white bars) and cells exposed to BAC (black bars).

Furthermore, a different temperature could be used that represents the conditions in the food industry more closely. As can be seen in Fig. 6.1 some *S. aureus* strains are much stronger biofilm formers at 15 °C than at 25 °C.

It would be interesting to study whether the test developed with *S. aureus* would perform equally well with biofilms from other bacteria. For Gram-negative bacteria the

obvious candidate would be *P. aeruginosa*, because it is used as a representative of the Gram-negatives in the current standard European tests [9, 10]. Other Gram-negative bacteria could be the ones that cause problems in specific products, such as *Salmonella enteritidis* or *Escherichia coli*. For Gram-positive bacteria an obvious candidate is *L. monocytogenes*. A considerable number of biofilm studies have focussed on *L. monocytogenes* [27, 74, 101, 111, 121, 130, 143, 159, 166]. However, it has been questioned whether pure cultures of *L. monocytogenes* are able to form a biofilm. It was suggested that *L. monocytogenes* may use primary colonizers to form a biofilm [101, 159]. If this is the case, such a primary colonizer can be used together with *L. monocytogenes* for mixed biofilm formation. However, at least some *L. monocytogenes* strains are able to form biofilms at 25 °C and/or at 15 °C (Fig. 6.2) that are comparable in thickness to that of *S. aureus* as determined by the saffranine method described in Fig. 6.1. The difference in biofilm formation between Scott A and its resistant variant Scott A Nis^R [174] clearly demonstrates the effect of *L. monocytogenes* strain diversity on biofilm formation.

Finally, the viability assessment by plate counting in the test could be replaced with fluorescent labeling. This would shorten the protocol with 2 days. Fig. 6.3 shows that it is possible to use fluorescent labeling in combination with flow cytometry instead of plate counting for viability assessment of *S. aureus* biofilm cells exposed to BAC. Fluorescent probes could also be used in combination with fluorescence microscopy together with phase contrast microscopy or confocal laser scanning microscopy of intact biofilms, which will give information about the spatial distribution of disinfectant killed cells. A fluorescent probe that has been used for this purpose in several studies is CTC [92, 131, 132]. However, CTC is not suitable for *L. monocytogenes* viability assessment after disinfection (Chapter 4) and also in other studies its suitability has been questioned [103, 161]. An alternative fluorescent method would be measurement of the internal pH of bacteria in the biofilm with CFSE. This probe has been used for biofilm analysis in combination with CLSM and two-photon excitation microscopy [177]. However, surrounding the biofilm cells with medium of a low pH, allowing for assessment of pH gradients, i.e. pH_{in} versus pH_{out}, may be a problem in thick biofilms. Another alternative may be one of the dye exclusion probes, but these probes should only be used for cells exposed to non-oxidative disinfectants (see Chapter 4). Furthermore, probes may also be used in combination to obtain more information (see below). Of course, once an alteration to the test has been made, one must keep in mind that the suitability of the biofilm quantification method and the reproducibility of biofilm formation and biofilm disinfection have to be validated again, because the alteration may influence biofilm formation.

Finally, standardized biofilms may be valuable tools to study the genes that are induced or repressed in biofilm cells compared to planktonic cells. Such studies may lead to the discovery of genes that are important for resistance of biofilms cells [181] and genes that code for targets for disinfectants in biofilms. The flow through system used in this thesis offers a good alternative for the batch biofilms that are mostly used for such studies.

VIABILITY ASSESSMENT AFTER EXPOSURE TO A DISINFECTANT

The traditional method to determine viability of bacteria, also in disinfectant testing, is plate counting. This method is based on the reproductive capacity of cells. However, this method has some disadvantages. The plate count technique requires long incubation times (2 days). Furthermore, for viability assessment of attached or biofilm cells, the cells have to be removed from the surface for analysis. Additionally, several studies report that cells are metabolically active while they are incapable of the cellular division required to form a colony on a plate. This is also known as a viable but non culturable (VBNC) state [32, 48, 102, 120] or better: an active but non culturable state (ANC) [100]. In the case of disinfectants this may lead to overestimation of the efficacy of the disinfectant.

In this thesis several other methods for viability assessment were used. Respiration, acidification and ATP-measurements (Chapter 2) are very rapid, give an indication of the energy status of the cells and the immediate effect of the disinfectant can be analyzed (< 1 min). Unfortunately, a high cell concentration (> 10^9 ml^{-1}) is needed to be able to detect a signal, thus the sensitivity of these methods is very low. Furthermore, these methods analyze the response of the total population and thus they can not be used to quantify viability. For hydrogen peroxide (HP) exposed cells these methods pose additional problems because of the production of large amounts of oxygen, i.e. gas bubbles which interfere with sample taking and proper functioning of measuring instruments. This problem may be overcome by removing the disinfectant from the cell suspension by washing before the measurements, but then the immediate effect of the disinfectant can not be analyzed.

Another rapid alternative for plate counting is fluorescent labeling of cells. At first a fluorimeter was used for analysis of fluorescent cells (Chapter 2). The advantage of this method is that the immediate effect of the disinfectant can be analyzed and followed during a chosen time period. The drawback of the method is that the total population is analyzed and a high cell concentration is needed (10^9 ml^{-1}). In further studies flow cytometry was used for analysis of fluorescently labeled cells (Chapter 4). The advantages of this method are that it is rapid and individual cells can be analyzed in great quantities (1,000 cells sec^{-1}). The disadvantages are that all intact cells are detected and thus large numbers of cells have to be analyzed to detect possible low numbers of viable cells among a large number of dead but intact cells. Furthermore, the sensitivity is not so high, the lower detection limit is a 2 to 3-log reduction depending on the signal to noise ratio [115]. Therefore, log 4 or log 5 reductions in viability are difficult to determine.

A general advantage of the above-mentioned alternatives to plate counting is that they show the reaction of the cells to a disinfectant at the sub-cellular level. These methods provide information on cell processes and cell properties that are affected at low and relatively harmless concentrations (such as loss of the proton motive force after exposure to BAC [Chapter 2] or complete loss of ATP after exposure to HP [data not shown]). They also show which processes or properties are affected at high concentrations that result in complete

loss of viability according to plate counts (such as loss of respiration and membrane integrity after exposure to BAC [chapters 2 and 4]) and which properties are not altered at these high concentrations and may indicate a viable but non culturable state (such as membrane integrity after exposure to HP [Chapter 4]). However, insight into disinfectant damage at the sub-cellular level does not allow conclusions to be drawn about cell viability as determined by plate counts. First it has to be known which of the damages to the cell caused by the disinfectant correspond with complete loss of viability according to plate counts, before a method can be selected as an alternative to plate counting. If the suitable killing concentration has to be determined for a disinfectant of which the precise mechanism of action is known, this choice will not be such a problem, but for screening of new disinfectants it will be. This problem could be solved in several ways:

First of all, the 'golden standard of viability', plate counting, could be abolished and replaced by another standard. In that case the use of the term 'viable but not culturable' or 'active but non culturable' will be redundant, but a similar term may have to be introduced for the newly accepted standard. Several authors suggest that a good new standard would be maintenance of an intact membrane, as shown by the exclusion of dye exclusion probes [38, 140]. However, membrane integrity is not always a good viability indicator. Cells that are non-viable according to several other methods may exclude dye-exclusion probes because the membrane is not damaged or damage is not extensive enough for dye exclusion probes to enter the cells, for example in HP exposed *L. monocytogenes* cells that exclude TOTO (Chapter 4) and for PI exclusion of *L. monocytogenes* exposed to HP (data not shown). Furthermore, cells without an intact membrane can be viable, for instance after electroporation or exposure to heat [141], or membrane integrity can be restored [176].

Another approach would be to use different alternative methods at the same time instead of plate counting, for instance double-labeling of cells with fluorescent probes. This approach will give multiple indices of physiological activity and will provide insight in the overall effect of a disinfectant on physiological activity and on the site(s) of sub-lethal injury [100, 119]. In short, there are perspectives for the use of fluorescent probes as an alternative for plate counting in disinfectant testing when the proper probes are selected. They can be used in combination with flow cytometry provided the cells can be easily removed from the surface and in combination with non-destructive methods such as confocal laser scanning microscopy if they are tightly bound to the surface.

BIOFILM CONTROL IN PRACTICE

There are several ways to control biofilm formation in practice. The best way of control is of course prevention of biofilm formation. Surfaces can be treated such that they repel bacteria, they can be coated with antimicrobials [112] or with substances that interfere with their quorum sensing system [80], they can be made out of material that leaches biocides or

antimicrobials can be attached covalently to the surface to prevent exhaustion of the antimicrobial [170]. Another method is to place a current over the surface or to allow a biofilm of harmless micro-organisms to develop that will competitively exclude pathogenic micro-organisms [112, 117, 118]. Most of these methods are expensive or developed for other purposes than the food industry, e.g. to prevent colonization of medical devices and implants. Thus, more research is needed before they can be applied in the food industry. A simpler and more effective method for biofilm prevention is to follow the guidelines of hygienic design of EHEDG [11]. Examples of these guidelines for hygienic design are use of smooth surfaces, avoidance of dead ends, corners and crevices in the used materials.

Despite all methods and precautions, biofilm formation can never be completely prevented [67]. Thus, biofilm formation should be monitored so that cleaning and disinfection of surfaces can be done at the proper time. For the monitoring of biofilm formation a large variety of techniques is available. In the most frequently used methods biofilms are first removed from the surface, for instance by swabbing or scraping [41, 88, 124]. Then cells can be detected and their viability can be determined by techniques such as ATP-measurements, plating and fluorescence labeling [88, 92, 124]. In the future new viability techniques based on the detection of mRNA [106, 142] may be used. Biofilm formation in pipe systems can be monitored without opening them by measurement of pressure change or current change in a small device that contains a large surface area for biofilm formation and receives the same liquid flow as the pipe system. These systems are already commercially available [7, 60]. Which of these techniques is preferable depends on the situation. With the data obtained from these measurements, biofilm build up can be monitored in time. Furthermore, with these data the efficiency of cleaning and disinfection can be assessed. With the help of the acquired data a model can be developed to estimate the appropriate time for cleaning and disinfection and a cleaning and disinfection regime can be developed accordingly [58].

For efficient cleaning and disinfection, it is not only important that the cells in the biofilm are killed. The biofilm matrix has to be broken down as well, so that the biofilm can be completely removed from the surface. Any left over organic material provides nutrients that facilitate the rapid formation of a new biofilm [186]. Biofilm matrix breakage, which also makes the biofilm cells better accessible for disinfection, can be done by a cleaning agent, an additional agent such as EDTA or by the disinfectant itself [186].

With the help of the biofilm test described in this thesis, known and candidate disinfectants, such as for instance electrolyzed water [104], can be evaluated for their biofilm killing efficacy. This evaluation will teach us which disinfectants are most effective against biofilms, which in the end will contribute to food safety and food quality and the control of cleaning costs in the food industry.

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Summary

Every year foodborne diseases cause millions of illnesses worldwide. The symptoms include diarrhea, nausea, vomiting and fever. In a limited number of cases there may be severe complications and sequelae such as meningitis, septicemia, arthritis, kidney disfunctioning and even death. It is therefore necessary to control the microbiological quality and safety of our food very strictly. Bacteria are always present on and in raw foods. Numerous processing techniques have been developed to kill or inactivate or prevent further growth of pathogenic and spoilage bacteria. However, when the bacteria from the raw food have been killed or inactivated food can still be recontaminated with bacteria. Thus, everything that comes into contact with food should be free of bacteria or have a level of contamination that is not harmful. This applies to all food contact surfaces and processing equipment in the food industry. Therefore, these surfaces have to be cleaned and disinfected on a regular basis to prevent accumulation of pathogenic and spoilage organisms. Significant problems are posed by bacteria that can attach to these surfaces and form biofilms, since this mode of growth makes them less susceptible to disinfectants than free-living bacteria. In Europe disinfectants are traditionally tested on bacterial cell suspensions (free-floating cells) in so-called suspension tests. Thus, disinfectant testing would greatly profit from the inclusion of a biofilm disinfectant test. Furthermore, the disinfectant tests that are currently used employ plate counting for viability assessment of the survivors. This is a time-consuming method (2 days) and rapid alternatives would further improve the test. One such alternative is the use of fluorescent probes, which allows reduction of the time for viability assessment to approximately two hours.

The aim of the research in this thesis was to study the effect of disinfectants on free-living cells and biofilm cells. For this purpose different methods were used to study the effect of disinfectants on the cell level and factors that influence the response of cells to disinfectants. In addition, we wanted to improve the current European disinfectant tests. To achieve this goal we studied factors that influence the efficacy of disinfectants and alternatives for viability assessment by plate counting. Furthermore, we developed a biofilm disinfectant test. For this study the human pathogenic bacteria *Staphylococcus aureus* and *Listeria monocytogenes*, which are both known biofilm formers, were used.

In Chapter 2 the difference in susceptibility of *L. monocytogenes* exponential- and stationary-phase cells to benzalkonium chloride (BAC) was determined by plate counting and the results were linked to relevant bio-energetic parameters, such as respiration, ATP-content and proton motive force. At a low concentration of BAC (8 mg l^{-1}) a similar small reduction in viable cell numbers was observed for exponential phase cells and stationary-phase cells (approximately 0.22 log unit reduction), although their membrane potential and pH gradient were dissipated. However, at higher concentrations of BAC, exponential phase cells were more susceptible than stationary-phase cells. At 25 mg l^{-1} the difference in survival on plates

was more than 3 log units. For both types of cells killing, i.e. more than one log unit reduction in survival on plates, coincided with complete inhibition of acidification and respiration, and total depletion of ATP pools. Factors that did not influence killing efficiency were the presence of glucose, BHI medium or oxygen. The results of this chapter suggest that growth phase is one of the major factors that determine the susceptibility of *L. monocytogenes* to BAC.

In Chapter 3 the influence of growth phase on the resistance of *S. aureus* to the surface-active agents BAC and dodecylbenzyl sulphonic acid (DSA) and the oxidizing agents sodium hypochlorite (NaOCl) and hydrogen peroxide (HP) was studied. The resistance of cells in different growth phases was compared to that of solid medium cells grown according to the European phase 1 suspension test, that is used to test disinfectants. Using cells from different growth phases with the same concentration of colony forming units per milliliter we showed that decline-phase cells were the most resistant cells. However, the decline-phase cell suspension contained more than 90 % dead cells. A 10-fold diluted suspension with a total concentration of cells equal to that of the other cell suspensions still revealed decline-phase cells generally to be the most resistant cell type. However, the resistance was drastically reduced indicating that the large proportion of dead cells provided a significant protection to the viable decline-phase cells. HP resistance of decline-phase cells could be partly explained by the high catalase activity in the dead cell fraction. Exponential phase cells were less resistant than decline-phase cells and, surprisingly, stationary-phase cells were the least resistant of the three. Cells grown according to the European phase 1 suspension test were never the most resistant cells. Their survival was 1 to 3 log units less than that of the most resistant cells. This chapter shows that the solid medium cells currently used are not the most resistant type of cells that can be used in a disinfectant test.

In Chapter 4 we compared fluorescent labeling methods in combination with flow cytometry with plate counting as indicators for viability of *L. monocytogenes* cells exposed to BAC and HP. Furthermore, we studied the influence of growth phase on fluorescent labeling and susceptibility to disinfectants. The fluorescence methods used were labeling with CTC (dehydrogenase activity), labeling with TOTO (membrane impermeant probe) and assessment of the maintenance of a pH-gradient in a low pH buffer after labeling with the pH-sensitive probe CFSE (pH_{in}-method). Growth phase influenced fluorescent labeling, but not to such an extent that it influenced the way in which the distinction between viable and non-viable cells was made. Growth phase also influenced susceptibility to disinfectants. As was found in Chapter 2, stationary-phase cells were less susceptible to BAC than exponential phase cells. However, stationary-phase cells were more susceptible to HP than exponential phase cells. The viability of cells exposed to BAC determined with plate counts correlated well with CTC-labeling and TOTO-exclusion. The viability of BAC exposed and HP exposed cells determined by plate counts did not correlate quantitatively with the pH_{in}-method. However, the pH_{in}-method gave a good indication of viability, sub-lethal damage and cell death. CTC-labeling and TOTO-exclusion did not correlate with viability of HP exposed cells

determined by plate counts. Notably, at a HP concentration which reduced the number of colony forming units to 0.2 % of the original number, more than 92 % of the cells were classified viable based on exclusion of TOTO. Thus, provided the proper fluorescent probes are selected, flow cytometry can be used as a rapid alternative for viability assessment by plate counting.

In Chapter 5 a standardized disinfectant test for *S. aureus* cells in biofilms was developed. Two disinfectants, the membrane active BAC and the oxidizing agent NaOCl were used to evaluate the biofilm test. *S. aureus* formed biofilms on glass, stainless steel and polystyrene in a simple system with constant nutrient flow that mimicked as closely as possible the conditions used in the current standard European disinfectant tests (e.g. EN 1040). The biofilm that was formed on glass contained cell clumps and extracellular polysaccharides. The average surface coverage was 60 % and most of the biofilm cells (92 %) were viable. Biofilm formation and biofilm disinfection in different experiments were reproducible. For biofilms exposed to BAC and NaOCl a 50 and a 600 times higher concentration was needed, respectively, to achieve 4 log killing than for the cells from the European phase 1 suspension test. The results in this chapter show that a standardized disinfectant test for biofilm cells is a useful addition to the current standard tests.

Chapter 6 is a discussion of the results and gives some perspectives for the further development of the biofilm disinfectant test. For biofilm formation other strains or bacteria could be used instead of the currently used *S. aureus* DSM 799. It is shown in this chapter that several *S. aureus*- and *L. monocytogenes* strains are better able to form a biofilm at 15 °C and/or 25 °C than the currently used strain. Furthermore, it is shown that fluorescent labeling can be used as a rapid alternative to plate counting for viability assessment after exposure of biofilm cells to a disinfectant.

This study shows that growth phase and growth in a biofilm are factors that have a large influence on susceptibility to disinfectants. These factors should be taken into consideration for improvement of current disinfectant tests and development of new tests. This study also shows that fluorescent labeling in combination with flow cytometry can be used as a rapid indicator of cell viability after exposure of free-living cells and biofilm cells to disinfectants. Furthermore, a standardized biofilm disinfectant test was developed, which may be developed further according to the suggestions made in the General discussion (Chapter 6). With the help of the biofilm test, currently used and candidate disinfectants can be evaluated for their biofilm killing efficacy. This evaluation will show which disinfectants are most effective against biofilms, which in the end will contribute to food safety and food quality and also to control of the cleaning costs in the food industry.

Samenvatting

Elk jaar worden er in de wereld miljoenen mensen ziek door het eten van voedsel dat besmet is met pathogenen. De symptomen zijn onder andere diaree, misselijkheid, overgeven en koorts. In een klein gedeelte van de gevallen kunnen ernstige complicaties optreden, zoals hersenvliesontsteking, bloedvergiftiging, gewrichtontsteking, verstoring van de nierwerking en zelfs de dood. Het is dus erg belangrijk om de microbiologische kwaliteit en veiligheid van voedsel goed in de hand te houden. In rauw voedsel zijn altijd bacteriën aanwezig, dus zijn er in het verleden veel behandelingen ontwikkeld die pathogenen en bederfflora doden of er voor zorgen dat ze niet verder groeien. Maar ook na behandeling kan voedsel (her)besmet worden met bacteriën. Alles wat met voedsel in aanraking komt, moet dus bacterievrij zijn of een besmettingsgraad hebben die niet schadelijk is. Dit geldt voor alle oppervlakken en machines in de voedingsmiddelenindustrie waarmee voedsel in aanraking komt. Deze oppervlakken moeten dus regelmatig schoongemaakt en gedesinfecteerd worden om te voorkomen dat pathogenen en bederfflora zich ophopen.

Bacteriën die zich aan oppervlakken kunnen hechten en biofilms vormen, zijn een groot probleem. Bacteriën in biofilms zijn namelijk minder gevoelig voor desinfectantia dan vrijlevende bacteriën. In Europa worden van oudsher bacteriesuspensies gebruikt om desinfectantia te testen, in zogenoemde suspensietesten. Een biofilm desinfectietest zou dus een goede aanvulling zijn op de al bestaande testen.

In de desinfectietesten die op dit moment gebruikt worden, bepaalt men de levensvatbaarheid van de overlevende bacteriën met behulp van plaattellingen. Bij deze methode duurt het minstens twee dagen voordat de uitslag bekend is, dus een sneller alternatief zou de testen kunnen verbeteren. Een voorbeeld van zo'n alternatief is het gebruik van een fluorescerende stof. Bij deze methode is de uitslag al na ongeveer twee uur bekend.

Het doel van het onderzoek in dit proefschrift was tweeledig. Ten eerste wilden we meer inzicht krijgen in het effect van desinfectiemiddelen op pathogenen, vrijlevend of in een biofilm. Daarom hebben we met verschillende methoden bekeken wat er in de cellen gebeurt na blootstelling aan desinfectiemiddelen en welke factoren daar invloed op hebben. Het andere doel was om the huidige Europese desinfectietesten te verbeteren. Met dit doel hebben we factoren die de effectiviteit van desinfectantia beïnvloeden bekeken en alternatieven voor bepaling van levensvatbaarheid met behulp van de plaattelmethode. Verder hebben we een biofilmtest ontwikkeld. De bacteriën die we bij dit onderzoek gebruikt hebben zijn *Staphylococcus aureus* en *Listeria monocytogenes*. Van deze twee bacteriën is bekend dat ze biofilms kunnen vormen.

In Hoofdstuk 2 is de gevoeligheid van *L. monocytogenes* exponentiële- en stationaire-fase cellen voor benzalkonium chloride (BAC) bepaald met behulp van plaattellingen. De resultaten hiervan werden vergeleken met metingen aan parameters die belangrijk zijn voor de energiehuishouding van de cel, zoals ademhaling, ATP-gehalte en membraanpotentiaal.

Bij blootstelling aan een lage BAC-concentratie (8 mg l^{-1}) werd een zelfde afname (ongeveer 0.22 log-eenheden) in kolonievormende eenheden (kve) voor exponentiële- en stationaire-fase cellen waargenomen. Bij hogere BAC-concentraties waren exponentiële-fase cellen gevoeliger dan stationaire-fase cellen. Bij 25 mg l^{-1} was het verschil in kve groter dan 3 log-eenheden. Bij allebei de celtypen viel afdoding, meer dan 1 log-eenheid afname in kve, samen met volledige remming van verzuring en ademhaling, en totale uitputting van de ATP-voorraad. Factoren die geen invloed hadden op de afdoding waren de aanwezigheid van glucose, BHI-medium of zuurstof. De resultaten uit dit hoofdstuk geven aan dat groeifase een van de belangrijkste factoren is die de gevoeligheid van *L. monocytogenes* voor BAC bepalen.

In Hoofdstuk 3 werd de invloed van groeifase op de resistentie van *S. aureus* tegen de oppervlakte-actieve stoffen BAC en dodecylbenzyl sulfonzuur (DSA), en de oxiderende stoffen natrium hypochloriet (NaOCl) and waterstofperoxide (HP) onderzocht. De resistentie van cellen in verschillende groeifasen werd vergeleken met die van cellen, die gekweekt waren op vast medium volgens de voorschriften van de Europese fase 1 suspensietest voor desinfectantia. Wanneer we bij blootstelling van de cellen in verschillende groeifasen evenveel kve per milliliter gebruikten voor elk celtype, vonden we dat cellen in de afstervingsfase het meest resistent waren. De suspensie met afstervingsfasecellen bevatte echter meer dan 90 % dode cellen. Toen deze suspensie tien keer verdund werd, wat er voor zorgde dat de totale celconcentratie even hoog was als die van de andere celsuspensies, bleek dat de afstervingsfasecellen over het algemeen nog steeds het meest resistente celtype waren. De resistentie was wel fors lager, wat een aanwijzing is dat de dode cellen die in de suspensie met afstervingsfasecellen zitten, flinke bescherming geven aan de levende cellen. De resistentie van afstervingsfasecellen tegen HP kon gedeeltelijk verklaard worden door de hoge catalase-activiteit van de dode celfractie. Exponentiële-fase cellen waren minder resistent dan afstervingsfasecellen, en, heel verrassend, stationaire-fase cellen waren het minst resistent van de drie. Cellen die volgens de voorschriften van de Europese suspensietesten gekweekt waren, waren nooit het meest resistent. Hun overleving was altijd 1 tot 3 log-eenheden lager dan die van de meest resistente cellen. Dit hoofdstuk maakt duidelijk dat de op vast medium gekweekte cellen die op dit moment gebruikt worden voor desinfectietesten, niet het meest resistente celtype zijn dat gebruikt kan worden in een desinfectietest.

In Hoofdstuk 4 hebben we kleuring door fluorescente stoffen in combinatie met flowcytometrie vergeleken met plaattelingen voor het bepalen van de levensvatbaarheid van *L. monocytogenes* cellen die blootgesteld waren aan BAC en HP. Verder hebben we de invloed van groeifase op fluorescente kleuring en op de gevoeligheid voor desinfectantia bekeken. De fluorescente methoden die gebruikt werden, waren: CTC-kleuring (dehydrogenase activiteit), TOTO-kleuring (stof die niet doorgelaten wordt door de intacte celmembraan) en een methode waarbij de handhaving van de pH-gradient gemeten werd in een buffer met lage pH, met behulp van de pH-gevoelige stof CFSE (pH_{in}-methode). De

groeifase beïnvloedde de fluorescente kleuring, maar niet zoveel dat de manier waarop het onderscheid gemaakt werd tussen levensvatbare of niet-levensvatbare cellen beïnvloed werd. De groeifase beïnvloedde wel de gevoeligheid voor desinfectantia. Zoals we ook al hadden gevonden in Hoofdstuk 2, waren stationaire-fase cellen minder gevoelig voor BAC dan exponentiële-fase cellen. Daarentegen waren stationaire-fase cellen gevoeliger voor HP dan exponentiële-fase cellen. De levensvatbaarheid van aan BAC blootgestelde cellen die bepaald was met behulp van plaattellingen, kwam goed overeen met de resultaten van de CTC-kleuring en de TOTO-uitsluiting. De levensvatbaarheid van aan BAC en HP blootgestelde cellen bepaald aan de hand van plaattellingen had geen kwantitatieve correlatie met de pH_{in}-methode. De pH_{in}-methode gaf wel goed aan of een cel levend, beschadigd of dood was. Bij cellen die blootgesteld waren aan HP, kwamen CTC kleuring en TOTO-uitsluiting niet overeen met de levensvatbaarheid bepaald door plaattelingen. Sterker nog, bij een HP concentratie die het aantal kve verlaagde tot 0.2 % van het oorspronkelijke aantal zou, afgaande op TOTO-uitsluiting, 92 % van de cellen nog levensvatbaar zijn. Alleen als dus de goede fluorescente stoffen geselecteerd worden, kan flowcytometrie gebruikt worden als een snel alternatief voor de bepaling van levensvatbaarheid met plaattellingen.

In Hoofdstuk 5 werd een gestandaardiseerde desinfectietest voor *S. aureus* cellen in biofilms ontwikkeld. Twee desinfectantia, het membraanactieve BAC en het oxiderende NaOCl werden gebruikt om de biofilmtest te evalueren. *S. aureus* vormde biofilms op glas, roestvast staal en polystyreen in een simpel systeem met een constante nutriëntenstroom, dat zoveel mogelijk de omstandigheden nabootste die in de huidige Europese desinfectietest gebruikt worden (bijv. EN 1040). De biofilm die op glas was gevormd, bevatte celklompjes en extracellulaire polysachariden. De gemiddelde oppervlaktebedekking was 60 % en de meeste biofilmcellen (92 %) leefden. Biofilmvorming en biofilmdesinfectie waren reproduceerbaar in verschillende experimenten. Om een 4 log afname in kve te krijgen in biofilms, was er voor BAC en NaOCl respectievelijk een 50 keer en een 600 keer zo hoge concentratie nodig als voor de Europese suspensietest cellen. De resultaten in dit hoofdstuk laten zien, dat een gestandaardiseerde desinfectietest voor biofilmcellen een nuttige toevoeging is aan de huidige standaardtesten.

Hoofdstuk 6 is een discussie van de resultaten en geeft enkele uitgangspunten voor het verder ontwikkelen van de biofilm desinfectietest. In plaats van *S. aureus* DSM 799, die nu wordt gebruikt, zouden andere stammen of bacteriën gebruikt kunnen worden voor biofilmvorming. In dit hoofdstuk wordt aangetoond dat verscheidene *S. aureus*- en *L. monocytogenes*-stammen betere biofilmvormers zijn bij 15 en/of 25 °C, dan *S. aureus* DSM 799. Verder wordt aangetoond dat fluorescente kleuring gebruikt kan worden als een snel alternatief voor de bepaling van levensvatbaarheid met plaattellingen, na blootstelling van biofilmcellen aan desinfectantia.

Deze studie laat zien dat groeifase en het groeien in een biofilm factoren zijn die de gevoeligheid voor desinfectantia sterk beïnvloeden. Met deze factoren moet rekening gehouden worden bij het verbeteren van bestaande desinfectietesten en het ontwikkelen van

nieuwe testen. Ook wordt er aangetoond dat fluorescente kleuring in combinatie met flowcytometrie gebruikt kan worden als een snel alternatief voor de bepaling van levensvatbaarheid met plaattellingen, na blootstelling van vrijlevende cellen en cellen in biofilms aan desinfectantia. Verder werd er een gestandaardiseerde biofilm desinfectietest ontwikkeld, die verder ontwikkeld kan worden volgens de suggesties uit de algemene discussie (Hoofdstuk 6). Met behulp van de biofilmtest kunnen bekende en nieuwe desinfectantia getest worden op hun werkzaamheid tegen biofilms. Hierdoor zullen we te weten komen welke desinfectantia het effectiefst werken tegen biofilms, wat uiteindelijk zal bijdragen aan de veiligheid en de kwaliteit van ons voedsel en de beheersing van de schoonmaakkosten in de voedingsmiddelenindustrie.

Samenvatting voor leken

Elk jaar worden er in de wereld miljoenen mensen ziek door het eten van voedsel, dat besmet is met ziekteverwekkende bacteriën.

Bacteriën zijn de kleinste levensvorm en ze bestaan uit één cel. De meeste bacteriën zijn zo klein dat je ze alleen met een microscoop kunt zien: ongeveer 1 micrometer oftewel 1/1000 mm. Bacteriën kunnen erg nuttig zijn voor mensen, denk bijvoorbeeld maar aan bacteriën die witte kool conserveren door er zuurkool van te maken of die melk omzetten in yoghurt. Of aan de bacteriën die aan de binnenkant van je darm zitten en je zo tegen andere, slechte bacteriën beschermen. Wanneer een bacterie ziekte kan veroorzaken, wordt deze een pathogeen genoemd. Bekende voorbeelden van pathogenen zijn *Salmonella*, die veel op kipproducten zit, en *Legionella* die in warmwatersystemen voorkomt. In dit proefschrift werden twee pathogenen die in voedsel kunnen voorkomen onderzocht, namelijk *Staphylococcus aureus* (zie Figuur 1.4) en *Listeria monocytogenes* (zie achterkant van de kaft). Wanneer je voedsel eet dat met *S. aureus* besmet is, kun je last krijgen van misselijkheid, buikkrampen en overgeven. Bij voedsel besmet met *L. monocytogenes* kunnen mensen met een zwak immuunsysteem soms last krijgen van hun maag-darmstelsel maar, veel erger, ze kunnen last krijgen van griepachtige symptomen die later kunnen overgaan in bloedvergiftiging, hersenvliesontsteking of ontsteking van de baarmoeder bij zwangere vrouwen, met alle gevolgen van dien voor moeder en kind.

Uit het bovenstaande blijkt dat het erg belangrijk is om er voor te zorgen dat er zo weinig mogelijk pathogenen in ons voedsel zitten. De moeilijkheid hierbij is dat op rauw voedsel altijd bacteriën zitten, waaronder soms ook pathogenen. Die pathogenen moeten dus zoveel mogelijk verwijderd of gedood worden. Dat kan bijvoorbeeld door rauw voedsel goed te wassen, het te koken, te zouten of te pasteuriseren. Maar dan nog kunnen er problemen ontstaan, omdat voedsel her-besmet kan worden met pathogenen. Dit kan bijvoorbeeld gebeuren doordat het al bereide voedsel in aanraking komt met een voorwerp of een oppervlak waarop zich door contact met andere besmette voorwerpen pathogenen bevinden. Het is thuis en zeker in de levensmiddelenindustrie dus ook heel belangrijk dat er zich op de voorwerpen, oppervlakken en apparaten waar voedsel mee in aanraking komt geen pathogenen bevinden. Een heel goede manier om hiervoor te zorgen is goed schoonmaken. Thuis doe je dat met een sopje en in de levensmiddelenindustrie doen ze dat door regelmatig het vuil te verwijderen met een schoonmaakmiddel en daarna te desinfecteren.

Voor het desinfecteren worden desinfectiemiddelen gebruikt. Deze middelen zorgen er voor dat een heel groot gedeelte van de aanwezige bacteriën gedood wordt. In deze studie zijn vier desinfectiemiddelen gebruikt. De eerste twee, benzalkonium chloride (BAC) en dodecylbenzeensulfonzuur (DSA) zijn ionische oppervlakte-actieve stoffen, die bijvoorbeeld in WC-reiniger zitten. De andere twee, natrium hypochloriet (NaOCl), een chloorverbinding, en waterstofperoxide (HP), zijn agressieve (oxiderende) stoffen. Deze stoffen maken bijna

alles kapot wat op hun weg komt. Voordat een middel officieel een desinfectiemiddel mag worden genoemd, moet het eerst getest worden. In Europa worden hiervoor gestandaardiseerde desinfectietesten gebruikt die opgesteld zijn door de Europese normalisatie-commissie. Een middel wordt bij deze testen pas goedgekeurd als desinfectiemiddel, wanneer het er voor zorgt dat er binnen vijf minuten van 10.000.000 levende pathogenen er nog maar 100 overblijven (99.999 % doding).

Desinfectiemiddelen worden normaal gesproken getest op bacteriën die in water rondzweven. Dit heet een bacteriesuspensie. Dit is eigenlijk heel erg vreemd, want in de voedingsmiddelenindustrie groeien bacteriën niet in suspensie maar op oppervlakken. Ze hechten zich aan het oppervlak en gaan zich dan heel snel vermenigvuldigen, laagjes vormen en ze scheiden een soort slijm af. Het resultaat hiervan noemen we een biofilm. Het is algemeen bekend, dat bacteriën in een biofilm op een of andere manier resistenter (= beter bestand) zijn tegen slechte omstandigheden dan bacteriën in een suspensie. Onder deze hogere resistentie valt ook de resistentie tegen desinfectiemiddelen. Het zou dus goed zijn om desinfectiemiddelen niet alleen te testen op bacteriën in suspensies, maar ook op bacteriën in een biofilm. Maar dan moet er wel een desinfectietest voor biofilmbacteriën ontwikkeld worden, want die bestaat nog niet.

Het doel van dit onderzoek was het verbeteren van de bestaande Europese desinfectietesten. Daarvoor hebben we gekeken naar factoren die invloed hebben op de effectiviteit van desinfectiemiddelen en naar snelle manieren om te bekijken of een bacterie gedood is door een desinfectiemiddel of niet. Ook hebben we een desinfectietest voor biofilmbacteriën ontwikkeld.

We zijn dit onderzoek begonnen met het bekijken van het effect dat het desinfectiemiddel BAC heeft op *L. monocytogenes* cellen, die we op twee verschillende manieren hebben laten groeien in een suspensie (Hoofdstuk 2). Het bleek dat cellen in de exponentiele fase (cellen die snel groeiden) veel minder goed tegen BAC konden dan cellen in de stationaire fase (cellen die bijna niet meer konden groeien door gebrek aan voedingsstoffen). Dit was ook wat we verwachtten, want cellen die zich in slechte omstandigheden bevinden wapenenen zich daar tegen en kunnen daardoor vaak ook beter tegen andere slechte omstandigheden, zoals blootstelling aan desinfectiemiddelen. Verder hebben we nog met speciale methoden gekeken wat het nou precies is dat BAC doet, waardoor de cel doodgaat. Het blijkt dat BAC gaten maakt in het omhulsel van de cel en dat daardoor allerlei celbestanddelen naar buiten lekken zodat de cel dood gaat.

Een volgend onderdeel van het onderzoek was om te kijken naar het effect van de vier bovengenoemde desinfectiemiddelen op *S. aureus* cellen (Hoofdstuk 3). Dit keer lieten we ze op drie verschillende manieren groeien in een suspensie. Ook gebruikten we *S. aureus* cellen, die we precies zo hadden laten groeien als er voorgeschreven staat in de Europese desinfectietest. Het bleek dat cellen die zeven dagen geen voedsel gehad hadden (cellen in de afstervingsfase), het beste tegen alle desinfectiemiddelen konden. Dat was wat we verwachtten, want deze cellen bevinden zich natuurlijk in heel erg slechte omstandigheden en

hebben zich hiertegen heel goed gewapend, waardoor ze heel goed tegen andere slechte omstandigheden kunnen. Daarna waren de cellen in de exponentiele fase het meest resistent en het zwakste waren de cellen in de stationaire fase. Dit was verassend, want voor *L. monocytogenes* hadden we het omgekeerde gevonden (zie boven). De cellen van de Europese desinfectietest bleken bijna net zo slecht tegen de vier desisinfectiemiddelen te kunnen als de stationaire fase cellen, dus ze waren relatief zwak. De Europese desinfectietest zou dus verbeterd kunnen worden, door cellen te gebruiken die resistenter zijn dan de cellen die er nu voor gebruikt worden.

Een ander onderdeel van het onderzoek was het zoeken van een methode om snel te weten te komen of cellen nog leven na blootstelling aan desinfectiemiddelen (Hoofdstuk 4 en Figuur 6.3). Normaal worden hiervoor de cellen op een voedingsbodem (agarplaat) uitgespreid. Een cel die leeft gaat zich dan heel snel vermenigvuldigen zodat er na twee dagen een hoopje cellen zichtbaar is op de agarplaat. Dan kun je dus het aantal overlevenden tellen en deze methode heet dan ook de plaattelmethode. Een snellere methode waar je maar ongeveer twee uur voor nodig hebt, is het gebruik van fluorescerende (lichtgevende) stofjes. Als een cel met zo'n stofje gekleurd wordt, geeft dat aan dat hij een bepaalde eigenschap heeft die nodig is om te overleven, bijvoorbeeld dat hij nog ademhaalt of energie kan vrijmaken. Of een cel gekleurd is kun je met een microscoop zien of met een speciaal apparaat dat flowcytometer heet. We hebben gekeken of je de fluorescente stofjes kunt gebruiken in plaats van de plaattelmethode, voor *L. monocytogenes* cellen die blootgesteld zijn aan BAC of aan HP. Sommige fluorescente stofjes blijken goed te werken en andere niet. Dus als je een goed stofje uitkiest, kun je inderdaad al binnen twee uur zien of een desinfectiemiddel de cellen heeft doodgemaakt of niet. Op deze manier zou je dus ook de Europese desinfectietesten kunnen verbeteren, doordat je nu sneller resultaten kunt verkrijgen.

Het laatste onderdeel van het onderzoek was het maken van een desinfectietest voor biofilmcellen (Hoofdstuk 5). We hebben hiervoor *S. aureus* gebruikt, omdat die normaal ook gebruikt wordt in de Europese desinfectietesten. We hebben de biofilms laten groeien op kleine dunne schijfjes glas, die in een klein bakje geplaatst waren (Figuur 5.1). In Figuur 5.2 en op de voorpagina kun je zien hoe de biofilms er uit zagen. We vonden na 24 uur telkens ongeveer hetzelfde aantal levende cellen op de schijfjes. Na blootstelling aan BAC of NaOCl waren er telkens ongeveer evenveel levende cellen over. Dit is heel belangrijk, want als je telkens een andere uitkomst krijgt heb je natuurlijk niet zoveel aan een test. Zoals we al verwacht hadden, waren *S. aureus* cellen die in een biofilm groeiden heel resistent tegen desinfectiemiddelen. Het bleek dat we voor doding van 99.99 % van de biofilmcellen een 50 keer zo hoge concentratie BAC nodig hadden als voor de cellen van de Europese desinfectietesten. Bij NaOCl hadden we zelfs een 600 keer zo hoge concentratie nodig. Een desinfectietest voor biofilm cellen is dus een goede aanvulling op de bestaande testen.

Samengevat, er zijn uit dit onderzoek drie manieren gekomen om de officiële Europese desinfectietesten te verbeteren: ten eerste zouden er in de toekomst cellen gebruikt

kunnen worden, die resistenter zijn dan de cellen die nu in de testen gebruikt worden. Ten tweede zou je de testen sneller kunnen maken door fluorescente stofjes te gebruiken in plaats van de plaattelmethode. Als laatste zou je onze desinfectietest voor biofilmcellen toe kunnen voegen aan de officiële Europese desinfectietesten. Met deze biofilmtest kunnen we dan kijken welke desinfectiemiddelen de biofilmcellen het snelst en meest effectief dood maken en kunnen we testen of er nieuwe desinfectiemiddelen zijn die nog beter werken tegen biofilms. Deze kennis zal bijdragen aan de veiligheid en de kwaliteit van ons voedsel en aan de beheersing van de schoonmaakkosten in de voedingsmiddelenindustrie.

Nawoord

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Curriculum vitae

Suzanne Bernardina Ida Luppens werd op 30 oktober 1972 in Geldrop geboren. Ze groeide op in Nuenen en volgde vanaf 1985 haar middelbare onderwijs aan het Augustinianum in Eindhoven. Daar haalde ze in 1991 haar Gymnasium B diploma en in datzelfde jaar begon ze aan haar studie Bioprocestechnologie aan de Landbouwuniversiteit Wageningen. Haar studie sloot ze af met onderzoek bij de vakgroep Algemene Microbiologie (Dr. S. J. W. H. Oude-Elferink en Dr. A. J. M. Stams) en bij de vakgroep Virologie (Dr. J. G. M. Heldens en Prof. R. W. Goldbach). Haar stage liep ze bij de Vakgroep Geography and Environmental Engineering van de Johns Hopkins University in Baltimore, Verenigde Staten van Amerika (Dr. N. D. Durant en Dr. E. J. Bouwer). In 1996 behaalde zij haar doctoraal diploma. In 1997 startte ze met een gecombineerd promotieonderzoek bij de Leerstoelgroep Levensmiddelenhygiëne en -microbiologie en de Leerstoelgroep Consumententechnologie en Productgebruik van de Wageningen Universiteit, dat gesponsord werd door de Nederlandse Vereniging van Zeepfabrikanten. Hierin werd ze eerst begeleid door Prof. J. Oosterom en Dr. T. Abee en later door Prof. F.M. Rombouts en Dr. T. Abee. De resultaten van het onderzoek zijn beschreven in dit proefschrift. Vanaf 1 maart 2002 is zij werkzaam als Universitair Docent bij de afdeling Cariologie Endodontologie Pedodontologie, Faculteit der Tandheelkunde, Universiteit van Amsterdam.

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List of abbreviations

BAC	Benzalkonium chloride
BHI	Brain heart infusion
CFDA,SE	Carboxyfluoresceindiacetate succinimidyl ester
CFSE	Carboxyfluorescein succinimidyl ester
CFU	Colony forming units
CTC	5-Cyano-2,3-ditolyl tetrazolium chloride
DSA	Dodecylbenzyl sulphonic acid
FCM	Flow cytometry
HP	Hydrogen peroxide
NaOCl	Sodium hypochlorite
O.D.	Optical density
PBS	Phosphate buffered saline
TOTO	Quinolinium, 1-1'-[1,3-propanediylbis[(dimethyliminio) -3,1-propanediyl]]bis[4-[(3- methyl-2(3H)-benzothiazolylidene) methyl]]-, tetraiodide
TSA	Tryptone soya agar
TSB	Tryptone soya broth