Keywords
Disinfectant-testing
Food hygiene
Dairy industry
DVG-guidelines
Reference substances

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Determination of the bactericidal activity of chemical disinfectants against bacteria in dairies according to the DVG-guidelines

Summary

Background: In the Federal Republic of Germany the guidelines of the German Veterinary Society (DVG guidelines, 2007) for evaluating chemical disinfectants for use in the food industries are widely regarded as an appropriate procedure for the comparison of various disinfectant products and recommended use concentrations. This study was undertaken to evaluate the bactericidal activity of four disinfectant compounds which serve as reference disinfectants in the DVG-guidelines for the testing of disinfectants for use in dairies and the food industries.
Methods: In accordance with the DVG-guidelines, Gram positive and Gram negative bacteria (*Staphylococcus aureus* (ATCC 6538), *Enterococcus hirae* (ATCC 10541), *Pseudomonas aeruginosa* (ATCC 15442), *Escherichia coli* (ATCC 10536)) were used as test organisms as they are representative for causing contaminations in dairies. Formic acid, peracetic acid, glutaraldehyde and benzyl-alkyl-dimethyl ammonium chloride were used as test disinfectants. Suspension tests and surface tests were carried out according to the DVG guidelines. In order to simulate practical conditions as closely as possible, low fat milk was used as organic load.
Results: As expected, the results showed that higher concentrations and prolonged exposure times were necessary when test organisms were dried onto the surface of steel disks (carrier tests) as they were when the organisms were in suspension (suspension test). Differences in their susceptibility to the test disinfectants were noticed between the four strains of microorganisms. *S. aureus* was highly susceptible to formic acid, *E. hirae* was sensitive to peracetic acid, glutaraldehyde and benzyl-alkyl-dimethyl ammonium chloride, while *P. aeruginosa* was highly resistant to glutaraldehyde and *E. coli* was susceptible to glutaraldehyde.
Conclusion: These findings emphasize the need for caution in selecting an appropriate disinfectant for use on contaminated surfaces in dairies and in the dairy industry particularly in the presence of organic material (milk) as well as the need to include reference substances in the disinfectant testing procedure to be able to evaluate the bactericidal activity. Reference substances are an indispensable prerequisite for an objective comparison of the activity of different products and for an in-process verification of the susceptibility of the test organisms used.
Hyg Med 2008; 33 [11]: 463–471

Introduction

One of the main challenges faced by the dairy industry is prevention of contamination. Pathogens or spoilage bacteria must be prevented from entering raw milk and dairy products. To that effect, the microbial load must be limited. That applies for all surfaces coming into contact with foodstuffs, e.g. milk machines, accessories and dairy equipment.

Among the bacteria than can present a problem to raw milk and other dairy products are *Streptococcus agalactiae* and other streptococcal strains, coliform bacteria, *Pseudomonas strains, Corynebacterium pyogenes, Staphylococcus aureus, Bacillus cereus, Listeria monocytogenes, Yersinia enterocolitica, Salmonella strains, Escherichia coli* O157:H7 and *Campylobacter jejuni*.

To address hygiene measures in the dairy industry, the European directive 93/43/EEC introduced a new approach to quality control: the Hazard Analysis and Critical Control Points (HACCP) concept. While the directive sets out few special requirements, it does define rules on, inter alia, the topic of cleaning and disinfection. Disinfectants are selected according to several criteria: compatibility with the

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surfaces to be disinfected, economic feasibility, safety in the workplace, toxicological safety and biological degradability. But the microbicidal properties are the most important criterion.

The disinfectants most commonly used in the foodstuffs industry in Europe are quaternary ammonium compounds, hypochlorite, amphoteric compounds and peroxide [1]. Other disinfectants used include alcohols, aldehydes, phenolic compounds and chlorhexidine [2].

To evaluate disinfectants, standard tests permitting verification and comparison of the various substances are needed. These tests must be robust, tailored to everyday practices and acceptable at international level. Since it is difficult and expensive to conduct practical tests under everyday use conditions, disinfectants are generally evaluated on the basis of laboratory tests [3].

To investigate the efficacy of disinfectants designated for use in the dairy industry, for many years now there have been in place DIN / EN standards which had been formulated by Technical Committee TC 216 (Chemical Disinfectant and Antiseptic) of the European Standardisation Committee (CEN) [4,5]. Pursuant to the DIN / EN standards, efficacy testing is not based only on suspension tests, but also on germ carrier tests. The latter include quantitative determination of viable organisms recovered from a contaminated and dried surface without, and after, using the test disinfectant [3].

Up till 2007 in Germany the guidelines of the German Veterinary Society (DVG) for testing disinfectants for use in the foodstuffs industry stipulated a quantitative suspension test to determine the recommended application concentrations [6]. While the test methods took account of the practical conditions prevailing at the actual site of use, they did not take the DIN /EN methods into consideration. In the dairy industry the currently recommended concentrations for disinfectants are often based on the results of suspension tests carried out in the laboratory; their suitability for evaluating the bactericidal activity on biofilms must be critically appraised. While suspension tests do permit assessment of the bactericidal activity of disinfectants under a number of circumstances, they do not permit any insights into a disinfectant's actual efficacy when used on contaminated surfaces [7].

In 2007 the DVG published revised guidelines for testing the efficacy of disinfectants in the foodstuffs industry (DVG Guidelines, 4th edition, 2007) [8]. These revised guidelines now include quantitative efficacy tests based on European standards, while at the same time retaining the advantages conferred by the former DVG test method.

But one of the disadvantages of these DIN /EN standards resides in the fact that their test methodology does not make provision for any reference substances. However, these are important because it is only by using such reference substances that reliable comparison of the efficacy of commercially available disinfectants can be made and any changes in the sensitivity of the test organisms identified over time (internal control). Accordingly, the DVG has incorporated such reference substances into its updated guidelines. These must be selected in accordance with the principle active ingredient of the disinfectant to be tested and must be tested in parallel to the actual test disinfectants. In line with that approach, formic acid has been included as a reference substance for organic acids, glutaraldehyde for aldehyde compounds and peracetic acid as reference substance for oxidizing compounds. Benzyl alkyl dimethyl ammonium chloride is to be used as a reference substance for quartary ammonium compounds, amphosurfactants, cationic surfactants and biguanides.

The present study was thus conducted to test the bactericidal efficacy of formic acid, glutaraldehyde, peracetic acid and benzyl alkyl dimethyl ammonium chloride, i.e. to test the bactericidal efficacy of those references substances which have now been incorporated into the revised DVG guidelines for testing disinfectants in the dairies and in the foodstuffs industry The aim of the study was to collect data, and thus establish a databank, and devise a potential yardstick for comparing the efficacy of disinfectants, for assessment of the sensitivity of test organisms and, as such, for validation of test guidelines on the efficacy of chemical disinfectants.

Materials and Methods

Test organisms

Tests were performed to verify what action the reference substances had on four bacterial species: *Staphylococcus aureus* (ATCC 6538) and *Enterococcus hirae* (ATCC 10541) as representatives for the Gram-positive bacteria encountered in the dairy setting as well as *Pseudomonas aeruginosa* (ATCC 15442) and *Escherichia coli* (ATCC 10536) as the most important Gram-negative pathogenic bacteria which have been designated as test organisms pursuant to the DIN / EN standards and the DVG guidelines.

Challenge substance

As challenge substance 100 g/l skimmed milk was used to simulate the practical conditions in dairies.

Disinfectant substances

The following reference substances were used as disinfectant substances for the suspension and germ carrier tests: 98 % formic acid, 15 % peracetic acid, 50 % glutaraldehyde and 100 % benzyl alkyl dimethyl ammonium chloride (quartary ammonium compounds).

Test methods

To assess the bactericidal efficacy of the respective reference substances, as specified by the DVG guidelines [8], the suspension test (VIII. 1. 6. 2: "Suspension test for measurement of the inactivation kinetics using a protein challenge based on the MPN method)" and the germ carrier test (VIII. 1. 7. 2: "Germ carrier test as basis for listing") were conducted in each case. Each disinfectant substance was used in at least three concentrations, with at least one of them being within the active and one in the inactive range of the disinfectant substance. To that effect, the disinfectant was diluted in each case to the required concentration, using water of standardised hardness (WSH). Depending on the disinfectant substance, the following neutralizing agents were used: dinatrium hydrogen phosphate (Na_2HPO4) 0.2 mol (28.4 g/l) for formic acide, sodium thiosulphate 0.3 % (3 g/l) for peracetic acid, histidine 1.0 % (10 g/l) for glutaraldehyde and a mixture of polysorbate 80 (30.0 g/l), saponin (30,0 g/l),lecithine (3.0 g/l) and histidine (1.0 g/l) for benzyl alkyl dimethyl ammonium chloride . The neutralizing agents were selected on the basis of experiences gained in the laboratory.

For validation purposes, the minimum inhibitory concentrations (MICs) were determined as per the DVG guidelines

and additional validation tests were performed in parallel to the suspension and germ carrier tests [8].

Suspension test

The suspension test (VIII.1.6.2 "Suspension test for recording the inactivation kinetics using a protein and based on the MPN method") is the test method, based on the most probable number (MPN), used to measure the inactivation kinetics using challenge substances. The test principle is based on the suspension test specified in DIN / EN 1276 for evaluation of the bactericidal efficacy of chemical disinfectants in the foodstuffs industry. However, in contrast to the DIN / EN standard, the DVG guidelines specify exposure times of 5, 15, 30 and 60 min, and for identification of surviving test organisms the MPN method is used. Each 1 ml bacterial suspension was set to between1.5 x 108 and 5.0 x108 cfu/ml (spectral photometer and McFarland standard 70900) and 1 ml challenge was added. The mixture was kept for 2 minutes ± 10 seconds at 20 ± 1 °C. Then 8 ml of the 1.25-fold preconcentrated disinfectant solution was added and incubated for 5, 15, 30 and 60 minutes at 20 ± 1 °C. On expiry of the exposure time one aliquot was withdrawn and its bactericidal activity immediately neutralised or suppressed. To that effect, 1 ml of this mixture was transferred to a test tube containing 8 ml of the respective specific neutralizing agent, dissolved in 30.0 g/l TSB (trypton soya broth) and 1 ml water. After 5 minutes ± 10 seconds neutralisation duration at 20 ± 1 °C, 1 ml of the neutralised test mixture (composed of the neutralizing agent, disinfectant solution, challenge substance and bacterial suspension) was immediately withdrawn and dilutions of between 10- and 7-fold were prepared and the neutralised mixture and each of the dilution stages were evaluated using the MPN method. To that effect, for each dilution step three samples, each of 1 ml, were withdrawn and transferred to test tubes with 9 ml TSB plus the specific neutralizing agent. After incubating for three days at 37 ± 1 °C the number of surviving bacteria was quantified. This was based on the MPN table [9]. In parallel, tests were performed for validation of dilution neutralisation as well as a water control test. The reduction in the number of test organisms was calculated, compared with the water control, and this was done for each test organism, disinfectant concentration and exposure time. To quantify the number of surviving test organisms as per the MPN method, the results obtained for the positive and negative test tubes were recorded for each day. Following that, using the numeric combination given in a standardised MPN table, the most probable number of test organisms per volume unit of the original sample was determined. The reduction was calculated on the basis of the difference between the logarithm of the microbial count (cfu/ml) of the water control test and the logarithm of the microbial count of the test suspension after exposure to the disinfectant. At least three independent test series were performed. The disinfectant passed the suspension test if, under the selected test conditions, it had reduced the respective test organism by at least 5 log levels.

Germ carrier test

The germ carrier test, which served as the basis for a disinfectant qualifying for inclusion on the approved list of disinfectants as per the DVG guidelines, is based on the germ carrier test specified in DIN / EN 13697. This is a quantitative germ carrier test for assessment of the bactericidal efficacy of chemical disinfectants in the foodstuffs industry. Aliquots of 1 ml bacterial suspension were set at between 1.5 x 108 and 5.0 x 108 cfu/ml (spectral photometer and McFarland standard 70900) and 1 ml challenge substance was added. The mixture was kept for 2 minutes ± 10 seconds at 20 ± 1 °C. Stainless steel disks, steel quality 304 and with a 2 cm diameter and polished on both sides to grade 2 B, were used as test surfaces. The stainless steel carriers were placed horizontally in an open petri dish. They were then inoculated with 0.05 ml of the prepared test suspension (test organism suspension and challenge) and placed in an incubator for between 45 and 55 minutes at 37 °C until they were visibly dry. After drying, the disks were allowed to cool down at room temperature for around 5 to 10 minutes.

The time needed to cool down to room temperature is based on tests carried out in advance of the actual test series and on laboratory experience [10,11]. Following this, the inoculum was coated with a 0.1 ml layer of the disinfectant solution. For the water control tests, water of standardised hardness was used instead of the disinfectant solution. After expiry of the exposure times of 5, 15, 30 or 60 minutes, each germ carrier was transferred to its own bottle, containing 10 ml of a suitable neutralizing agent and around 5 g glass beads. After a 5-minute neutralisation time decadic dilution series were prepared in a trpyptone NaCl solution. Then the number of surviving bacteria was counted, as described above using the spread-plate method and the MPN method. In parallel, tests were performed for validation of dilution neutralisation as well as a water-based control test. The microbial count reduction was determined per test organism, disinfectant concentration and exposure time compared with the water control test. The disinfectant had passed the germ carrier test if the investigated test organism had been reduced by at least 4 log levels under the selected test conditions and within the selected exposure times.

Results

Reference substance: formic acid

E. hirae was the limiting test organism on using formic acid as reference substance. Using 3% formic acid, the 5 log level reduction required in the suspension test was reached with an exposure time of 30 minutes, and on using 2% formic acid at 60 minutes. The 4 log level reduction required in the germ carrier test was reached with 3 % formic acid and an exposure time of 15 minutes (Figure 1b). Lower concentrations and shorter exposures sufficed to inactivate Gram-negative test organisms. The most sensitive organism was *P. aeruginosa*: already a concentration 0.5 % at 30 minutes in the suspension test and of 1 % at 30 minutes in the germ carrier test were enough to assure a reduction by 5 log and 4 log levels, respectively (Figure 1c). The results for *S. aureus* and *E. coli* were between those values obtained for the two test organisms mentioned. In the suspension test it was possible to reduce *S. aureus* by 5 log levels with 1 % formic acid and with an exposure time of 30 minutes; for *E. coli*, 0.5 % and 60 minutes were needed. In the germ carrier test *S. aureus* was effectively reduced with a 1 % concentration and 30 minute exposure time (Figure 1a) and *E. coli* with a 1 % concentration and 60 minute exposure time (Figure 1d).

Reference substance: peracetic acid

S. aureus was the limiting test organism on using peracetic acid as reference substance. In the suspension test, a 5 log level reduction was achieved with 0.004 % peracetic acid and with 30 minutes exposure time, and with 0.001 % at 60 minutes. The 4 log level reduction required in the germ carrier test was reached with 0.050 % peracetic acid and an exposure time of 15 minutes and with 0.010 % within 30 minutes (Figure 2a). In the suspension test peracetic acid proved to be highly affective against *E. hirae* at concentrations of between 0.004 % and 0.01 % at 15 and 60 minutes, respectively. In the germ carrier test, it was possible to reduce the exposure time to 30 minutes using a concentration of on 0.010 % (Figure 2b). *E. coli* proved to be the most resistant of the Gram-negative bacteria. In the suspension test a 5 log level reduction was achieved with 0.004 % peracetic acid within 15 minutes, with 0.002 % and in 30 minutes and with 0.001 % in 60 minutes. In the germ carrier test 0.010 % peracetic acid was needed with an exposure time of 30 minutes (Figure 2d). The requisite reduction was achieved for *P. aeruginosa* in the suspension tests with 0.001 % peracetic acid and an exposure time of 60 minutes, whereas in the germ carrier test a 10-fold higher peracetic concentration was needed to assure the 4 log level reduction required (Figure 2c).

Reference substance: glutaraldehyde

On using glutaraldehyde as reference substance there were two limiting test organisms: *S. aureus* and *P. aeruginosa*. The 5 log level reduction required for these two test organisms was achieved with 1.5 and 0.5 % glutaraldehyde and with 30 and 60 minute exposure times. In the germ carrier test, 0.5% glutaraldehyde and 30 minute exposure time were needed to reduce these test organisms (Figures 3a,3b). Of the Gram-positive bacteria E. herae was the more sensitive, and *E. coli* was the more sensitive of the Gram-negative bacteria. In the suspension test the 5 log level reduction required was achieved for E. herae with 1.5% glutaraldehyde with 15 minute exposure time and with 0.5% with 60 minutes were needed. For the *E. coli* tests, 1.5% glutaraldehyde and 30 minutes or 0.5% and 60 minutes. In the germ carrier test the 4 log level reduction required was achieved for *E. hirae* and *E. coli* with 0.5 % glutaraldehyde within 30 minutes (Figure 3b, 3d).

Reference substance: Benzyl alkyl dimethyl ammonium chloride

Using benzyl alkyl dimethyl ammonium chloride as reference substance, *S. aureus* and *E. coli* were the limit test organisms. In the suspension test the 5 log level reduction required was reached for both test organisms with 3% benzyl alkyl dimethyl ammonium chloride in 30 minutes and with 1% in 60 minutes. In the germ carrier test, the 4 log level reduction required was achieved with concentrations of 3% in 15 minutes (*S. aureus*) or 30 minutes (*E. coli*) (Figures 4a, 4d). *E. hirae* and *P. aeruginosa* proved to be the organisms most sensitive to this reference substance. It was possible to reduce *E. hirae* counts by the requisite 5 and 4 log levels, respectively already with concentrations of 1 % in 30 minutes (suspension test) or in 60 minutes (germ carrier test) (Figure 4b). *P. aeruginosa* was reduced by the requisite 5 and 4 log levels, respectively, both in the suspension test and germ carrier test with 1 % Benzyl alkyl dimethyl ammonium

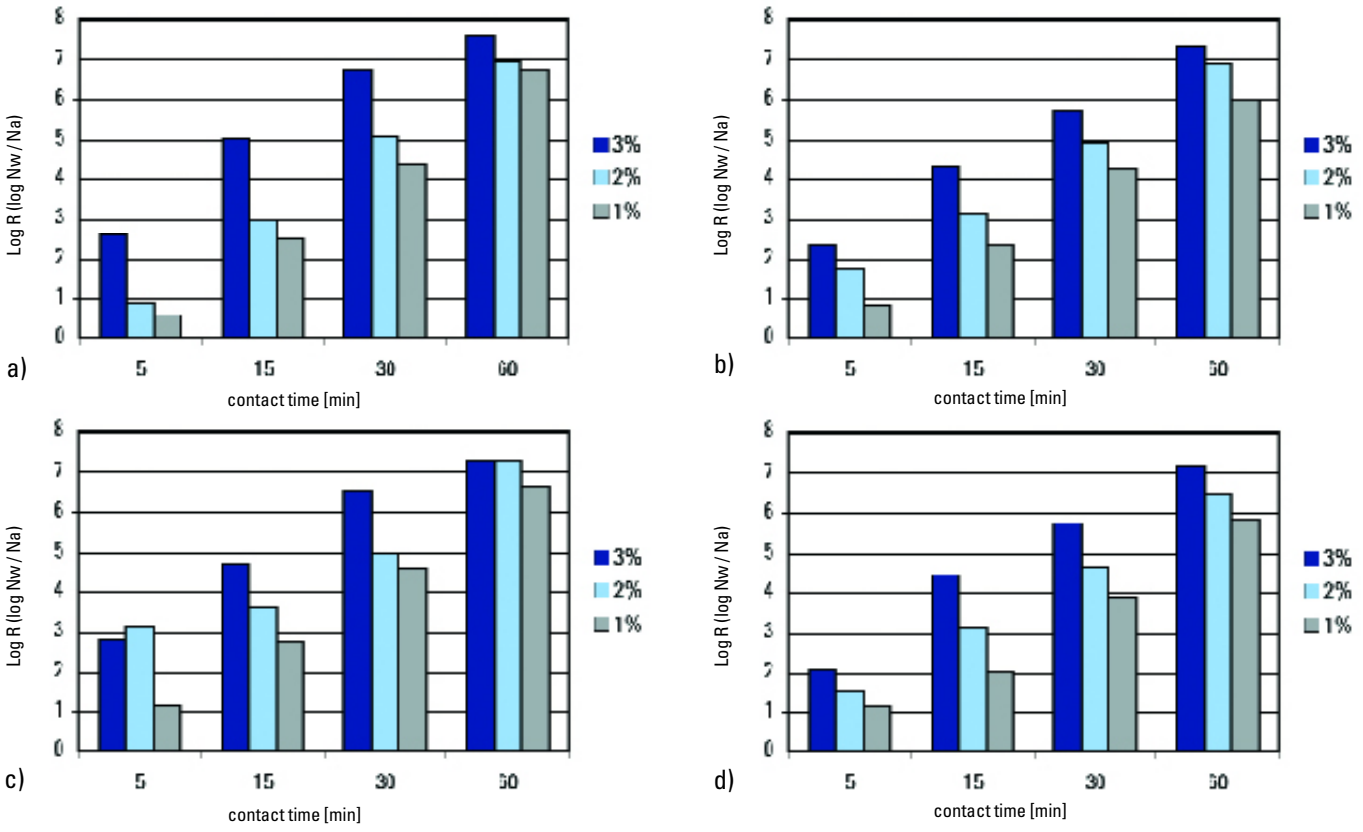

Figure 1: Mean log reductions using formic acid (germ carrier test). a) Staphylococcus aureus, b) Enterococcus hirae, c) Pseudomonas aeruginosa, d) Escherichia coli (log N_w = log of bacterial count for water control test; log N_a = log of bacterial count for test mixture.

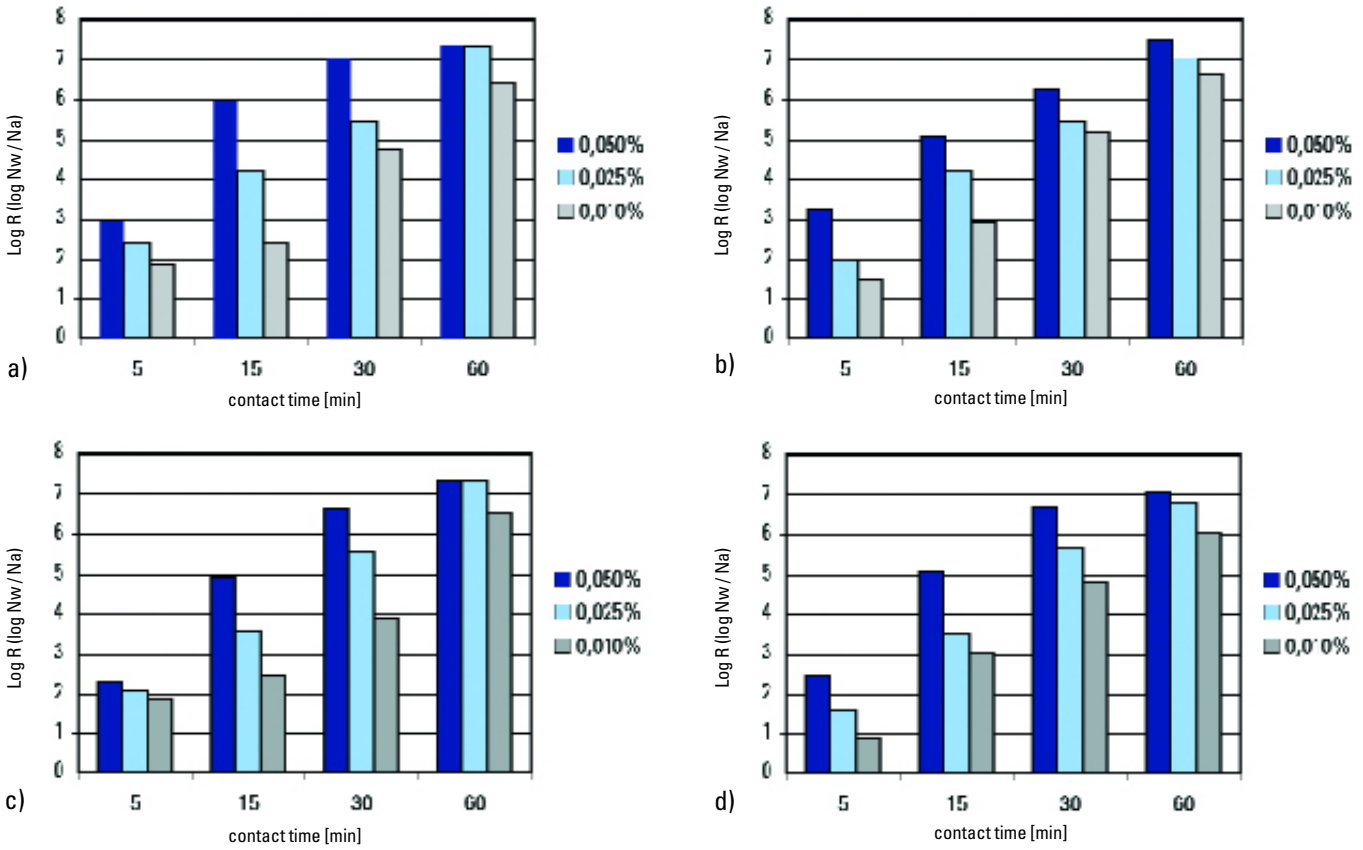

Figure 2: Mean log reductions using peracetic acid (germ carrier test). a) Staphylococcus aureus, b) Enterococcus hirae, c) Pseudomonas aeruginosa, d) Escherichia coli (log N_w = log of bacterial count for water control test; log N_a = log of bacterial count for test mixture.

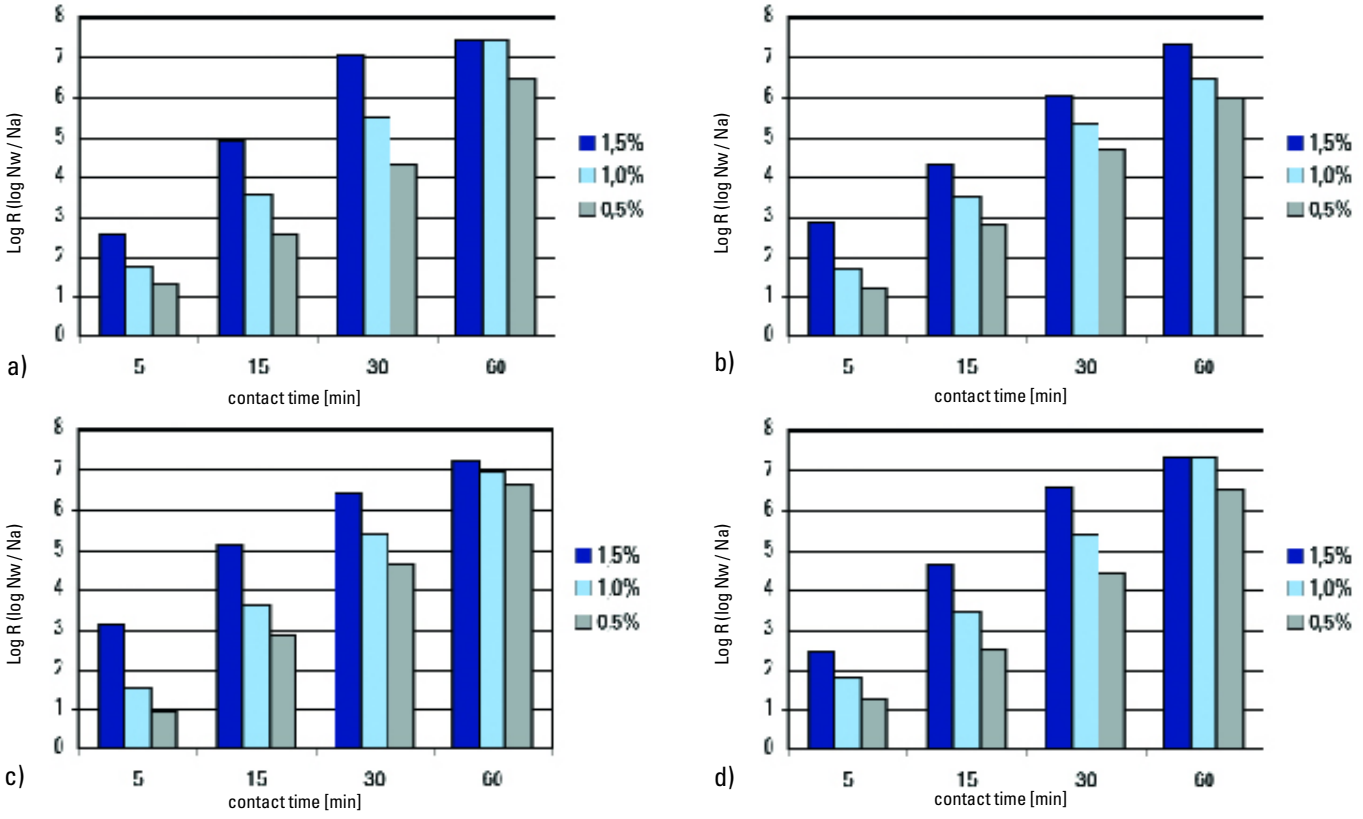

Figure 3: Mean log reductions using glutaraldehyde (germ carrier test). a) Staphylococcus aureus, b) Enterococcus hirae, c) Pseudomonas aeruginosa, d) Escherichia coli (log N_w = log of bacterial count for water control test; log N_a = log of bacterial count for test mixture.

chloride using a 60 minute exposure time (Figure 4c).

Discussion

Production of safe milk is accorded top priority in dairies. In that respect, risk analyses play a pivotal role. Hazards that could potentially undermine the quality and safety of milk or dairy products must be identified, anticipated at all stages of production and controlled. Only by adopting such an approach can contamination levels be reduced. In the dairy industry the Hazard Analysis and Critical Control Points concept is widely used. The risks encountered may be of a biological (bacteria, yeasts, moulds, parasites, dust), chemical (detergents, disinfectants) or physical (heat, pressure) nature. Hazard analysis should be conducted at an early stage of milk production and hygiene measures implemented. These include, in particular, cleaning and disinfection measures as well as the formulation of hygiene (infection control) policies. This approach confers several important advantages: 1. It ensures that no milk residues remain in the milk machines where they could have an adverse effect on product quality 2. It rules out contamination of dairy products with substances that could pose a hazard to the health of consumers; 3. The dairy equipment is cleaned at more frequent intervals, thus prolonging its service life, while reducing costs and increasing output. Dairies should use chemical disinfectants to kill microorganisms only if there are no suitable, e.g. biocide-free or physicial, alternatives for controlling pathogens.

Before being placed on the market, chemical disinfectants should be subjected to efficacy testing and should be registered, thus enduring that, when used as instructed, they are fit for purpose, will not have any unintended effects on the target species, will not give rise to any undesirable resistance or cause damage to humans, animals or the environment [12,13].

In 2007 the amended DVG guidelines for efficacy testing of the disinfectants used in the foodstuffs industry were published. To an extent, these are based on the methods specified in the DIN / EN standards, and also incorporate the latter, while at the same time retaining the advantages inherent in the former DVG test procedure. Unlike the DIN / EN standards, the DVG guidelines feature reference substances. Thanks to such reference substances, it is now possible to compare the efficacy of commercially available disinfectants and identify any changes occurring in the course of time in the sensitivity profiles of the test organisms (internal control). However, an extensive database is needed for objective evaluation. Only on the basis of such data can expert opinions on efficacy testing of chemical disinfectants be impartially validated and the efficacy of commercially available disinfectants be compared.

A further advantage conferrred by the DVG guidelines derives from the incorporation of the MPN method for quantification of surviving test organisms. The superiority of that method compared with the spread-plate technique, which is featured in the DIN / EN standards, has been investigated in several studies and its merits have been corroborated by the findings of the present study [14,15]. The MPN method is easy to use, poses few risks of contamination, nor it is time consuming or expensive. Growth of the test organisms can be observed more closely in liquid than on solid media and, if nec-

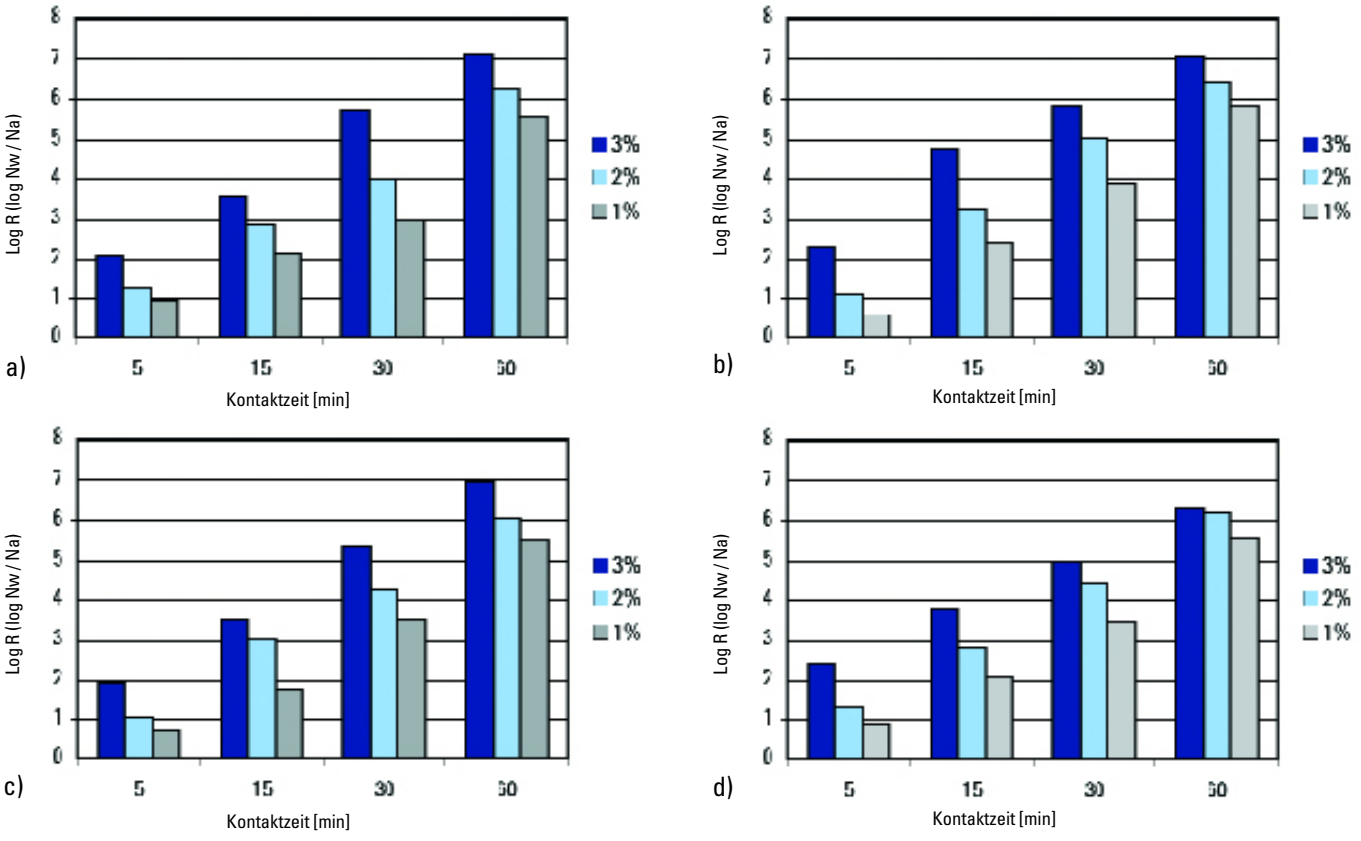

Abbildung 4: Mittlere Log-Reduktion bei Benzylalkyldimethylammoniumchlorid (Keimträgertest). a) Staphylococcus aureus, b) Enterococcus hirae, c) Pseudomonas aeruginosa, d) Escherichia coli (log N_w = log Keimzahl der Wasser-Kontrolle; log N_a = log Keimzahl des Prüfgemischs).

essary, the incubation duration can be greatly prolonged. As such, the test tubes can be incubated for more than three days without the medium drying out, while the test organisms are able to continue growing in the broth without any space constraints. Furthermore, a neutralizing agent specific to the respective nutrient medium is added to each test tube, thus ensuring that even sublethally damaged test organisms can multiply following neutralisation of any inadvertently transferred disinfectant residues. These observations concord with those of De Man [9], Black [16], Kamp et al. [14], Hunsinger et al. [17] and Rockhoff [15]. In the opinion of these authors, MPN method is particularly beneficial if liquid media are given preference over solid media, e.g. in the case of investigation of motile, swarming bacteria (e.g. Proteus mirabilis) or of test organisms that grow particularly slowly or fast or give rise to colonies that are difficult to count. This method is also ideal if the bacterial count in the samples is very low and, as such, no reliable yardstick can be defined for the population size. In such cases, a single colony can grow in the liquid medium after inoculation and is easier to detect in the test tube. Moreover, it provides for miniaturisation, whereby the requisite volume of the nutrient medium, including the test organism suspension, challenge substance and test disinfectant, can be reduced to a minimum by using minititre plates. In that way, one can devise a miniature form of the MPN method [15]. This reduces the time and resources invested, including the costs. Kleiner and Trenner [18] recommend this method for determination of the microbial count of surviving test organisms.

Suspension tests are relatively simple, do not call for any special or expensive laboratory techniques and, apart from the laboratory costs, they are inexpensive to conduct [3,7]. In addition, they are well standardised and therefore (within normal microbiological limits) they can be repeated, thus attesting to their reproducibility. This method can also be used to investigate how parameters such as exposure times, temperatures, test organisms and challenge substances affect disinfectant efficacy [19].

However, the greatest limitation of the suspension test is that it does not necessarily reflect the practical, everyday use conditions [19]. Some authors also do not consider it to be endowed with sufficient specificity as to rule out ineffective disinfectants [20]. Hence no application recommendations should be formulated on the basis of suspension test results. One exception to that is the recommendations for the Cleaning-in-Place (CIP) procedure.

The best insights into the efficacy of surface disinfectants in the laboratory tests are obtained from the germ carrier tests. These should reflect the everyday conditions prevailing in dairies and application recommendations can be formulated on the basis of such results [21].

The present study was aimed at identifying the bactericidal efficacy of four chemical disinfectants. These substances are representative of the active substances of disinfectants used in the dairy industry and are featured in the updated DVG guidelines as reference substances for the various disinfectant product groups. The need to select one reference substance per product group derives from the fact that it is only on using such an approach that the efficacy of various commercially available products can be compared with each other. Otherwise, the results obtained with reference substances can be used only to identify the sensitivity profiles of the test organisms [12,13]. All the disinfectant product groups tested are used in dairies. They are used for disinfection of tanks, containers, filters, mixing equipment, pipe systems, bottle centrifuges, pasteurisation systems, vaporiser equipment, etc. They are used to disinfect bottles (rinse water), for general cleaning of dairy equipment and fittings, as atomisers for air purification (bottle filling hall) and environmental hygiene. The findings of the current study demonstrated the following: In the suspension test for determination of the bactericidal efficacy of reference substances, *S. aureus* and *P. aeruginosa* proved to be highly sensitive to formic acid, whereas *E. coli* and, in particular, *E. hirae*, turned out to be resistant. The highest stability in respect of peracetic acid was exhibited by *S. aureus* and *E. coli*. Conversely, the two other test organisms used were highly sensitive to it. *E. coli* and *E. hirae* proved to be highly sensitive to glutaraldehyde, or at least shorter exposure times were needed to inactivate them compared with the other test organisms. The bactericidal effect exerted by benzyl alkyl dimethyl ammonium chloride was greater against *E. hirae* and *P. aeruginosa* than against *S. aureus* and *E. coli*, which required longer exposure times on using a similar concentration. As expected, in the germ carrier test longer exposure time and / or higher concentrations were needed in general than in the suspension test. Already in earlier studies, the relationship between, on the one hand, the germ carrier material, test organisms, challenge substance and drying time and, on the other hand, the results obtained in the germ carrier tests has been the subject of dicussion. Longer drying times (if the test suspension is allowed to dry on the steel disks for longer than 55 minutes) reduced in particular the Gram-negative but not the Gram-positive bacterial strains. That observation also concords with the findings of previous studies, including those of Reybrouck [22] who noted that Gram-negative bacteria were removed much faster from steel disks the longer they were allowed to dry. The same was concluded by Höller and Gundermann in their investigations into *P. aeruginosa* [23] as well as by Bloomfield et al., Van Klingeren and Hunsinger [3,11,24]. Nikaido and Vaara [25] as well Hirai [26] described the inherently high resistance evidenced by Gram-negative bacteria against various bactericidal substances, attributable to the presence of lipopolysaccharides in their outer membrane. However, due to the influence exerted also by the drying time, it has not been possible to fully investigate that. But by adding a challenge substance to the test system, it is possible to counter the lost drying activity, so that Gram-negative bacteria are reduced more slowly during the drying phase.

These findings concored with those of Hirai and Abele [10,27]. What is important is the choice of challenge substance and of germ carrier material, both of which have an impact on the test results [11,28,29,30,31]. That is borne out in particular on comparing the results of the present study, where stainless steel disks were used as germ carriers, with the findings of previous studies [11]. By adding skimmed milk as a challenge to the reference substances glutaraldehyde, formic acid and peracetic acid, shorter exposure times were needed, at the same concentration, for inactivation of the test organisms than when using a highly concentrated challenge substance (10 g/l yeast extract + 10 g/l bovine al-

bumin) as had been done in the studies conducted by Hunsinger [11]. Since in dairies and milk-processing units milk is likely to be the greatest source of contamination encountered, in the present study skimmed milk was used as a representative challenge substance for this area, as also specified in DIN / EN standards and DVG guideline. The test conditions were selected to simulate everyday use conditions, especially since the efficacy of disinfectants is reduced even by small concentrations of soils due to reactions with organic substances and this in turn detracts from the microbicidal activity of the disinfectant [13]. Apart from this reduction in the disinfectant effect, skimmed milk when used as a challenge appears to protect the test organisms, in particular Gram-negative bacteria, to a certain extent against the drying effects. That has been demonstrated on using formic acid against Gram-negative bacteria since higher concentrations were needed for the same concentrations. Higher concentrations or longer exposure times were also needed for peracetic acid and benzyl alkyl dimethyl ammonium chloride . In the case of peracetic acid the protective effect could also be noted for Gram-positive test organisms. This is attributable to the protein effect described for peracetic acid (inter alia, by Spicher and Peters [30]). In the presence of an organic substance and metal, peracetic acid disintegrates to water and oxygen. That process is further expedited in the presence of bacteria whose cell wall contains catalase, e.g. *S. aureus*. In the present germ carrier tests both metal and an organic substance, in the form of skimmed milk, are used, thus giving rise to a faster decline in the efficacy of peracetic acid. It is well known that disinfection is of paramount importance in dairies – both in terms of public health and economic considerations since appropriate disinfection measures will prevent microbial contamination of milk arising from working surfaces, equipment or other contact surfaces. On using methods such as the suspension or germ carrier tests to investigate disinfectants, the pros and cons of the different variations of these tests should be borne in mind. Moreover, each test should be viewed as only a component in a bigger test system since the ability of any one test on its own to provide valuable insights is relatively low [32]. Suspension tests alone are not enough to serve as a basis for formulation of application recommendations for disinfection practices in the dairy industry. They are suitable for use as screening tests to evaluate the effects exerted by various parameters, such as e.g. challenge substances and temperature, on the disinfectant outcome. But one should always bear in mind that microorganisms are in general resistant to biocides if they colonise a surface. That is all the more true if the organisms described here, together with a challenge substance, are to be found in a dry state on the surface. Disinfectant test methods do not take account of the microbial reduction resulting from the cleaning process, showing merely whether a disinfectant is endowed with antimicrobial properties when used in a suspension or on surfaces.

The results of such laboratory tests do not necessarily reflect the efficacy of disinfectants under the everyday use conditions, since other factors having implications for the disinfectant efficacy, such as temperature, can be simulated only to a certain degree in the laboratory. Hence germ carrier tests should be conducted under, as far as possible, realistic test conditions to simulate the real conditions, thus producing test results that can be used for formulation of application recommendations. Another consideration is the use of reference substances, which should be conducted in parallel to the actual test substances. Using such an approach, the stability of the test organisms can be verified and the efficacy of various commercially available disinfectants objectively compared with each other.

Conclusion

Our conclusions from the present study underline the need to incorporate the use of reference substances into the DVG test guidelines (2007). Only by using such an approach can the stability and/or resistance of the test organisms be investigated. Furthermore, various reference substances rather than just a single one, must be incorporated into the test guidelines to thus permit comparison of the efficacy of disinfectants. The reference substances selected as per the DVG guidelines can cover disinfectant substances whose use is not limited to the foodstuffs industry (and in particular to the dairy industry). They should therefore be viewed as general reference substances. The introduction of the MPN method for quantification of the most probable number of microbes present is also beneficial. This method is easy to use and cuts down on time, effort and costs, since fewer resources are needed for both conductance and evaluation of tests.

Conflict of Interest

The authors declare that there is no conflict of interest as understood by the International Committee of Medical Journal Editors.

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