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DETERMINATION OF THE EFFICACY OF CHEMICAL DISINFECTANTS AGAINST SOME GRAM POSITIVE AND GRAM NEGATIVE BACTERIA IN DAIRIES UNDER STANDARDIZED CONDITIONS (DVG-GUIDELINES)

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SUMMARY

In this study the bactericidal efficacy of four reference disinfectants used as standards in the recently published DVG-guidelines was assessed against Gram-positive and Gram-negative bacteria in dairies in the presence of organic matter (milk) by using two test methods according to the DVG-guidelines (2007) and European Standards which specify a test methods and minimum requirements for bactericidal activity of chemical disinfectants and antiseptics that are used in the dairies. This test methods are based on European standards (EN) which were prepared by the Technical Committee CEN/TC 216 (Chemical Disinfectant and Antiseptic).

The results showed that when we used suspension test which was the limiting test method for the listing of disinfectants for the food industries in the former DVG-guideline (2000) for determining the bactericidal efficacy of the tested reference disinfectants against tested organisms, *Staphylococcus aureus* (ATCC 6538) and Pseudomonas aeruginosa (ATCC 15442) were highly sensitive to formic acid while, *Escherichia coli* (ATCC 10536) and *Enterococcus hirae* (ATCC 10541) were more resistant. With application of peracetic acid the most resistant microorganisms were *Staphylococcus aureus* and *Escherichia coli*. While, the other two bacterial strains were highly susceptible. With glutaraldehyde the highly sensitive microorganisms were *Enterococcus hirae* and *Escherichia coli*. Benzyl-alkyl-dimethyl ammonium chloride showed higher bactericidal effect against *Enterococcus hirae* and Pseudomonas aeruginosa than against Staphylococcus aureus and *Escherichia coli* which needed longer exposure times at the same concentration.

So, The limiting test organism when using formic acid as reference substance was *Enterococcus hirae*. While, with peracetic acid application was Staphylococcus aureus. Both *Staphylococcus aureus* and Pseudomonas aeruginosa appear to be the limiting test organisms with glutaraldehyde. When using benzyl-alkyl-dimethyl ammonium chloride were *Staphylococcus aureus* and *Escherichia coli*.

Higher concentration and prolonged exposure times where necessary when test organisms were dried onto the surface of steel disks (carrier tests) as they were when the organisms were placed in suspension (suspension test) mainly with Gram negative organisms. This appears when using formic acid as reference substance against Gram negative test organisms we need higher concentrations in the same contact time. Also, with peracetic acid and benzyl-alkyl-dimethyl ammonium chloride applications higher concentrations respectively prolonged exposure time were required. This also was observed with Gram positive test organisms when using peracetic acid as reference substance. Differences in the disinfectant susceptibility were noticed between the four strains of microorganisms where, *Escherichia coli* was highly resistant to formic acid, while Pseudomonas aeruginosa was the most resistant strain to peracetic acid. Glutaraldehyde gave the same bactericidal effect against all tested strains. With benzyl-alkyl-dimethyl ammonium chloride the highly sensitive microorganism was *Enterococcus hirae*. These findings emphasize the need for caution in selecting an appropriate disinfectant for use on contaminated surfaces in dairies and 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 compare the activity of different products and check the susceptibility of the test organisms used.

INTRODUCTION

The general approach to hygiene in the milk industry has been changed by the publication of European Directive 93/43 of 14June 1993, with the adoption of a new approach to quality control taking into account HACCP-concept. It contains few specific requirements but general rules and among them the cleaning and disinfection procedures. The choice of suitable disinfectants depends on their compatibility with the surfaces to be treated, economical aspects, work safety issues, as well as their biodegradibility. The main aspect, will be the disinfectantís microbicidal properties. In the past, disinfectants were tested and validated by different methods within the European Union. The Federal Republic of Germany follows the guidelines of the German Veterinary Society (DVG, 4th edition, 2007) for evaluating chemical disinfectants for the use in the food industries. This currently published guidelines include quantitative efficacy tests based on European Standards (EN) developed by

the European Committee for Standardization (CEN) which is dealing with the task of coordinating the evaluation of disinfectants in Europe.

A main challenge in the food industry is to avoid contamination of raw materials and products by pathogens and spoilage organisms by controlling of microorganisms on food contact surfaces such as milking machine, milking utensils and dairy equipment.

Common problem-causing bacteria in the dairy industry are: Streptococcus agalactiae and other streptococci, coliform bacteria, *Pseudomonas spp., Arcanobacterium pyogenes, Staphylococcus aureus, Bacillus cereus, Listeria monocytogenes, Yersinia enterocolitica, Salmonella spp., Escherichia coli* O_{157}: H_7 and Campylobacter jejuni which represent bacterial pathogens of concern in raw milk and other dairy products. These microorganisms receive much attention from the scientific community as the general flora surviving good cleaning and disinfection routines until now.

The ideal disinfectant for this purpose should be of low toxilogical risk, no corrosion problems, compatibility with different technological surface materials, easily to rinse off without any residual problems, low ecological application as readily biodegradable, economical application and should not allow survival or growth of microorganisms. The most commonly used disinfectants in the food processing industry in European countries are quaternary ammonium compounds (QAC), hypochlorites, amphoteric compounds and peroxides (Wildbrett, 2002). Besides this, alcohols, aldehydes, phenolic compounds and chlorhexidine are also used (Holah et al., 2002).

For the evaluation of disinfectants, standard tests which are robust, relevant to use conditions and internationally acceptable are required to verify and compare activity. As field trials under use conditions are difficult and expensive to perform, the approval of disinfectants, for the most part is based on results of laboratory tests (Bloomfield et al., 1994). So far in the milk industries the recommended in-use concentrations of disinfectants are often based on laboratory suspension tests and one would not expect satisfactory bactericidal effect on biofilms. Although suspension tests can be used to assess the activity under a range of conditions, they give no information about how products actually perform on contaminated surfaces (Reybrouck, 1992). Meanwhile most of the EN-guidelines for assessing disinfectant activity of products intended to be used in the food industries are published. Beside in suspension tests these products have to prove efficacy also in surface tests. Surface tests involve quantitative determination of viable organisms recovered from a contaminated dried surface without and after application of the disinfectant (Bloomfield et al., 1994). Data concerning the disinfectant efficacy of commonly used disinfectant compounds are

not availably up to now. Also no reference substances are named which are chosen according the disinfectant compounds of the products to be tested. Therefore, this study was undertaken to evaluate the bactericidal activity of disinfectant compounds, which serve as reference disinfectants in the recently published DVG-guidelines (2007) in dairies and food industries against microorganisms which are representative, non pathogenic and covering tenacity and resistance of pathogens found in the field of application in order to set a data base and possibel standards for comparison of products.

MATERIALS AND METHODS

1-Strains:

Staphylococcus aureus (ATCC 6538); Enterococcus hirae (ATCC 10541); Pseudomonas aeruginosa (ATCC 15442) and Escherichia coli (ATCC 10536) were used as test organisms.

While, skim milk 100 g/L was used as interfering substance.

2-Disinfectants:

Formic acid 98 %; Peracetic acid 15 %; Glutaraldehyde 50 % and Benzyl-alkyl-dimethyl ammonium chloride 100 % with exposure times of 5; 15; 30 and 60 min were used for testing according to the DVG-guidelines. According the DVG-guidelines the reference substances tested in the actual tests should be chosen according to the main product compounds. Formic acid should serve as reference substance for organic acids; Glutaraldehyde covers the aldehyde compounds; Peracetic acid is used when oxidizing compounds should be tested, and Benzyl-alkyl-dimethyl ammonium chloride should serve as reference substance for quaternary ammonium compounds, amphotensides kationic tensides, and biguanids. Three product concentrations of each disinfectant were used which included at least one in the active and one in the non active range.

3-Neutralizers:

The neutralizers used in this study were Disodium hydrogen phosphate (Na_2 HPO_4), 0.2 mol (28.4 g/L) when using Formic acid as disinfectant; Sodium thiosulphate, 0.3 % (3 g/L) with Peracetic acid; Histidine, 1.0 % (10 g/L) with Glutaraldehyde and a mixture of Polysorbate 80, 30.0 g/L; Saponin, 30.0 g/L; Lecithin, 3.0 g/L and Histidine, 1.0 g/L in case of Benzyl-alkyl-dimethyl ammonium chloride. The neutralizer was chosen according to laboratory experience and validated using the MIC-Value determination according DVG-guidelines (2007) and in validation tests carried out in parallel to the suspension and surface test methods.

4-Test methods:

The test were performed according to the DVG-guidelines (2007) which include a suspension test and a surface test methods.

Suspension test: specifies a test method to determine the inactivation kinetics with interfering

substance using MPN-Method. The test method is based on the suspension test according to European Norms (EN) 1276 which specifies a quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectant used in the food industry. Differing from the EN standard 5, 15, 30 and 60 min exposure times were tested and MPN method was used to determine the viable counts. The principle of suspension test method was to dilute a sample of the product with water of standardized hardness and then add it to a mixture of test suspension of bacteria and interfering substance. 1 mL of a bacterial test suspension adjusted to 1.5 x10^8 to 5.0 x10^8 cfu/mL using Spectrophotometer and McFarland standard (REF 70 900) was added to 1 mL interfering substance. Skim milk was chosen as interfering substance in dairies with a final concentration of 100 g/L. The mixture was maintained at 20°C+1°C for 2 min +10 s. Then 8 mL of the product test solution were added and the mixture was maintained at 20°C+1°C for 5, 15, 30 and 60 min exposure time. At the end of the contact times an aliquot was taken and the bactericidal activity in this portion was immediately neutralized or suppressed by dilution-neutralization method adding 1 mL sample to a tube containing 8 mL of specific neutralizer dissolved in Tryptone Soya Broth 30.0 g/L and 1 mL water. After the neutralization time of 5 min +10 s at 20°C+1°C, a sample of 1 mL of the neutralized test mixture containing neutralizer, product test solution, interfering substance and test suspension was immediately taken and diluted with diluent to 10^7 dilution. Out of each dilution step the MPN method were carried out by taking 1 mL from each dilution step then inoculated in three broth tubes each one containing 9 mL Tryptone Soya Broth and specific neutralizer. After incubation at 37°C+ 1°C for 3 days the numbers of surviving bacteria in each sample were determined using the MPN-table (DE Man, 1983). In parallel tests for validation of the dilution neutralization and water control were carried out. For each test organism, product test concentration and exposure time, the reduction in viability in comparison to the water control was calculated.

For determination of the number of surviving test organisms according the MPN method the pattern of positive and negative tubes was noted each day and standardized MPN table was consulted to determine the most probable number of organisms per unit volume of the original sample. For calculation of the reduction for each test organism the number of cfu/mL in the bacterial test suspension and the test procedure was recorded and the decimal log reduction was calculated. This trial was performed 3 times using the previously mentioned bacteria as test organisms and previously mentioned disinfectant preparation as active compounds.

Surface test: according the DVG-guidelines is based on the surface test method described in EN 13697 which specifies a quantitative surface test

for the evaluation of bactericidal activity of chemical disinfectants used in the food industry. This test is using stainless steel discs with 2 cm diameter as test surfaces. The test was carried out with 100 g/L skimm milk serving as interfering substance. To prepare the test suspension two min. prior to the actual test 1 mL of the bacterial test suspension containing 1.5 $x10^9$ to 5.0 $x10^9$ cfu/mL was added to 1 mL of the interfering substance and mixed. The test surfaces were placed in an open petridish ensuring that the stainless steel discs were in horizontal position. Then they were inoculated with 0.05 mL of the test suspension and interfering substance mixture and dried in an incubator at 37°C for 45-55 min until they were visibly dry. After drying the temperature of the surface was adjusted to room temperature. Then the inoculum was covered with 0.1 mL of the product test solution, or for the water control with water of standardized hardness instead of the product. After the chosen exposure times of 5, 15, 30 and 60 min the surfaces were transferred into separate flasks containing 10 mL of an appropriate neutralizer and glass beads. After a neutralization time of 5 min a series of tenfold dilutions were prepared in Tryptone-NaCl solution. The number of surviving test organisms was determined quantitatively using MPN method as previously mentioned. In parallel tests validation of the dilution neutralization and water control were carried out. For each test organism, product test concentration and exposure time, the reduction in viability in comparison to the water control was calculated. The product was deemed to have passed the suspension test respectively the surface test if it demonstrated a 5 respectively 4 log reduction within the chosen contact times at 20°C or room temperature.

RESULTS

Results for the reference substance Formic acid

The limiting test organism when using formic acid as reference substance was Enterococcus hirae. The required 5 log reduction in the suspension test was achieved with concentrations of 3 % within 30 min and 2 % within 60 min exposure time, respectively. In the surface test 3 % formic acid was able to reduce the test organism on the steel carrier by 4 log within 15 min contact time (Figure 2). To inactivate the Gram negative test organisms lower concentrations respectively shorter exposure times were necessary. Most susceptible was Pseudomonas aeruginosa where a concentration of 0.5 % within 30 min in the suspension test and 1 % in 30 min in the surface test was able to reduce the test organisms by 5 log or 4 log, respectively (Figure 3). The test results of *Staphylococcus aureus* and *Escherichia coli* lay in between the results mentioned above. In the suspension test the required 5 log reduction was achieved with 1 % in 30 min for S. aureus and 0.5 % within 60 min exposure time for *E. coli*, while in the surface test 1 % in 30 min was necessary to inactivate *S. aureus* (Figure 1) and 1 %

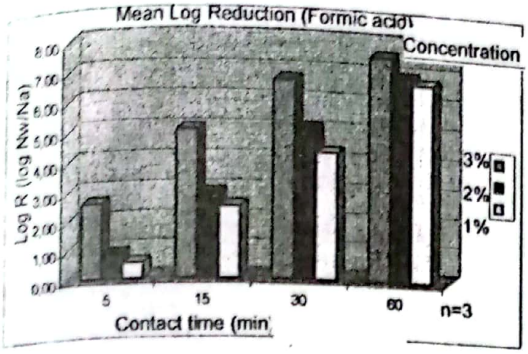

Figure 1: Mean log reduction of *Staphylococcus aureus* with formic acid in the surface test. Interfering substance 1% skim milk.

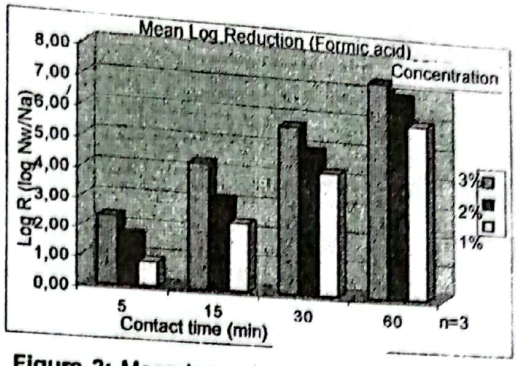

Figure 2: Mean log reduction of *Enterococcus hirae* with formic acid in the surface test. Interfering substance 1% skim milk.

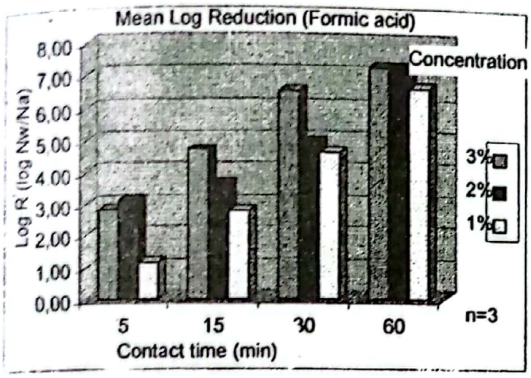

Figure 3: Mean log reduction of *Pseudomonas aeruginosa* with formic acid in the surface test. Interfering substance 1% skim milk.

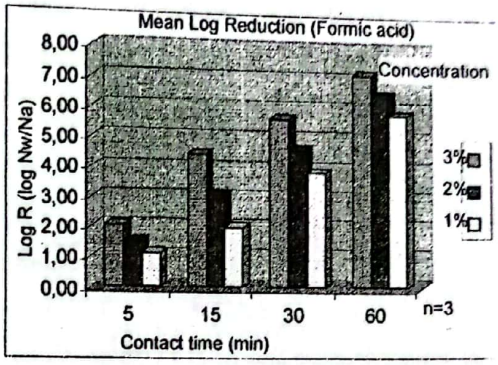

Figure 4: Mean log reduction of *Escherichia coli* with formic acid in the surface test. Interfering substance 1% skim milk.

Results for the reference substance Peracetic acid

When using peracetic acid as reference substance the limiting test organism was *Staphylococcus aureus*.. The number of the test organism was reduced to 5 log reduction by the application of peracetic acid with concentrations of 0.004 and 0.001 % within 30 and 60 min exposure time, respectively. While, on steel carrier it required higher concentrations as 0.025 and 0.010 % within 15 and 30 min exposure time, respectively to pass the 4 log reduction (Figure 5). Within a contact time of 15 and 60 min, respectively a concentration of 0.004 and 0.001 % peracetic acid was highly effective against *Enterococcus hirae* in the suspension test While, the required 4 log reduction was achieved in the surface test with concentration of 0.010 % in 30 min (Figure 6). The highly resistant Gram negative organism was *E. coli* because the required 5 log reduction in suspension test was obtained within exposure time of 15, 30 and 60 min with concentrations of 0.004, 0.002 and 0.001 %, respectively. In the surface test 0.010 % peracetic acid was necessary to inactivate the test organism within 30 min contact time (Figure 8). With a concentration of 0.001 % peracetic acid and exposure time 60 min the required reduction of Pseudomonas aeruginosa can be achieved meanwhile, the 4 log reduction was recorded in surface test within 60

min contact time by a concentration of 0.010 % (Figure 7)

Results for the reference substance Glutaraldehyde

Both *Staphylococcus aureus* and *Pseudomonas aeruginosa* appear to be the limiting test organisms with glutaraldehyde as reference substance. Glutaraldehyde by concentrations of 1.5 and 0.5 % yielded 5 log reduction of *S. aureus* and *P. aeruginosa* within exposure time 30 and 60 min, respectively. Glutaraldehyde was able to yield 4 log reduction of the previously mentioned test organisms on steel disk carriers by concentrations of 0.5 % after 30 min exposure time (Figure 9 and 11). Also, *Enterococcus hirae* and *E. coli*, respectively were the susceptible Gram positive and Gram negative test organisms where the 5 log reduction of suspension test was obtained within contact time 15 and 60 min with concentrations of 1.5 and 0.5 %, respectively in case of *Enterococcus hirae* while, for *E. coli* 1.5 and 0.5 % within 30 and 60 min, respectively. In the case of the surface test 0.5 % glutaraldehyde was able

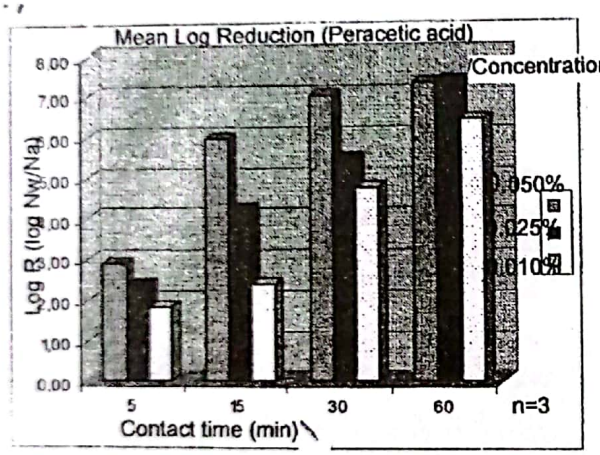

Figure 5: Mean log reduction of *Staphylococcus aureus* with peracetic acid in the surface test. Interfering substance 1% skim milk.

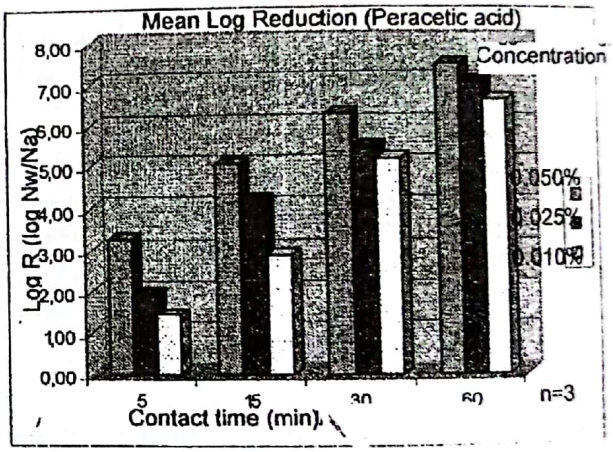

Figure 6: Mean log reduction of *Enterococcus hirae* with peracetic acid in the surface test. Interfering substance 1% skim milk.

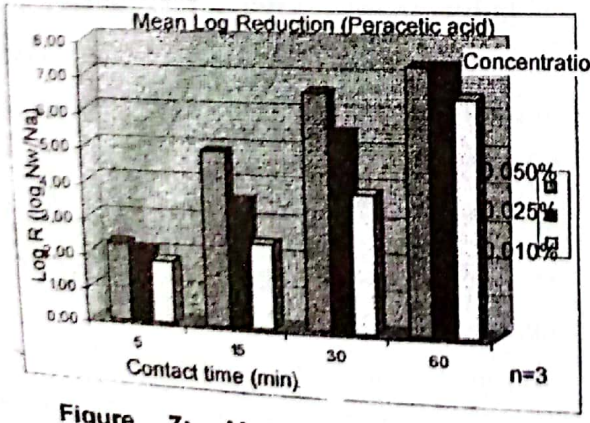

Figure 7: Mean log reduction of *Pseudomonas aeruginosa* with peracetic acid in the surface test. Interfering substance 1% skim milk.

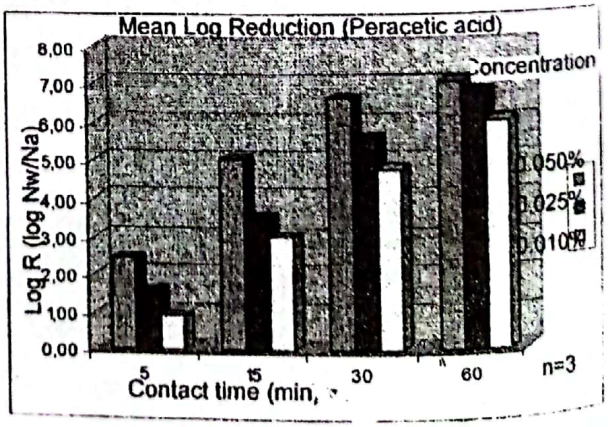

Figure 8: Mean log reduction of *Escherichia coli* with peracetic acid in the surface test. Interfering substance 1% skim milk.

to reduce the number of Ent. hirae and *E. coli* to 4 log reduction within 30 min contact time (Figure 10 and 12).

Results for the reference substance Benzyl-alkyl-dimethyl ammonium chloride (Quaternary ammonium compound)

The limiting test organisms when using benzyl-alkyl-dimethyl ammonium chloride as reference substance were *Staphylococcus aureus* and *Escherichia coli*. The required 5 log reduction in the suspension test was achieved with concentrations of 3 % within 30 min and 1 % within 60 min exposure time, respectively for the two limiting microorganisms. In the surface test 1 % benzyl-alkyl-dimethyl ammonium chloride was able to inactivate the test organisms on the steel carrier within contact time 60 min (Figure 13 and 16). Most sensitive microorganisms were *Enterococcus hirae* and *Pseudomonas aeruginosa* where a concentration of 1 % within 30 min in the suspension test and 1 % in 60 min in the surface test were able to reduce the number of *Enterococcus hirae* by 5 log or 4 log, respectively (Figure 14).

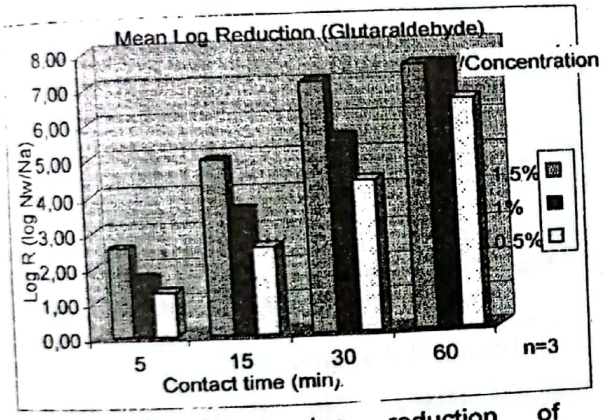

Figure 9: Mean log reduction of *Staphylococcus aureus* with glutaraldehyde in the surface test. Interfering substance 1% skim milk.

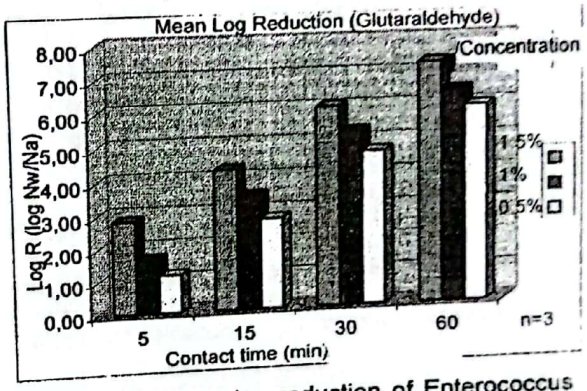

Figure 10: Mean log reduction of Enterococcus hirae with glutaraldehyde in the surface test Interfering substance 1% skim milk.

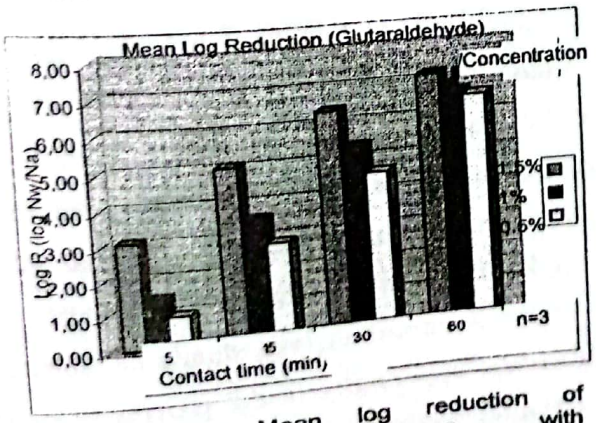

Figure 11: Mean log reduction of *Pseudomonas aeruginosa* with glutaraldehyde in the surface test. Interfering substance 1% skim milk.

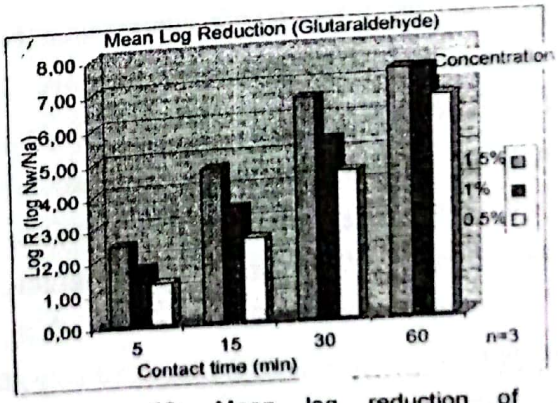

Figure 12: Mean log reduction of *Escherichia coli* with glutaraldehyde in the surface test. Interfering substance 1% skim

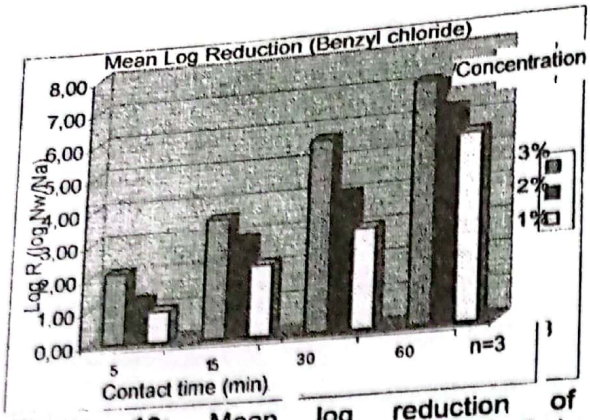

Figure 13: Mean log reduction of *Staphylococcus aureus* with benzyl-alkyl-dimethyl ammonium chloride in the surface test. Interfering substance 1% skim milk.

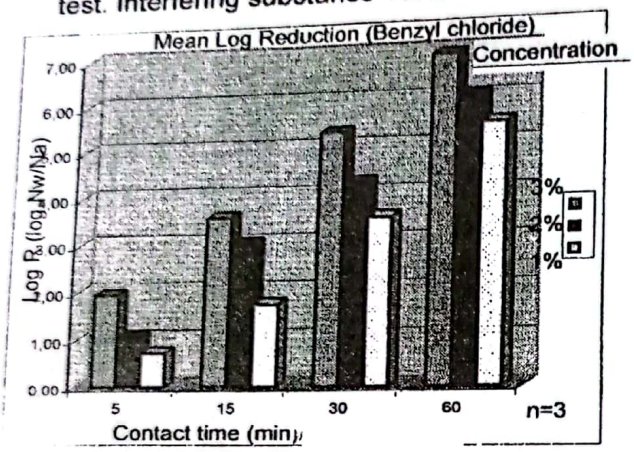

Figure 15: Mean log reduction of *Pseudomonas aeruginosa* with benzyl-alkyl-dimethyl ammonium chloride in the surface test. Interfering substance 1% skim

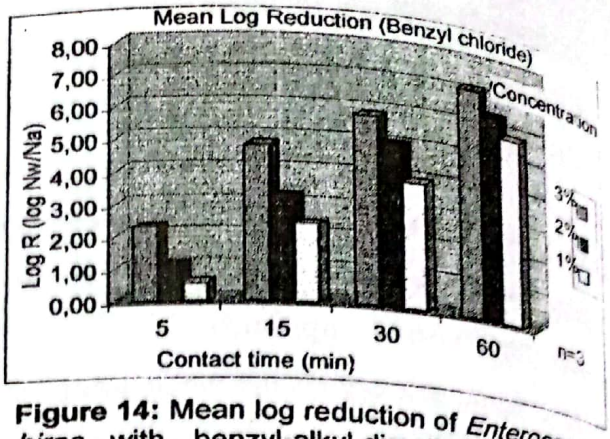

Figure 14: Mean log reduction of *Enterococcus hirae* with benzyl-alkyl-dimethyl ammonium chloride in the surface test. Interfering substance 1% skim milk.

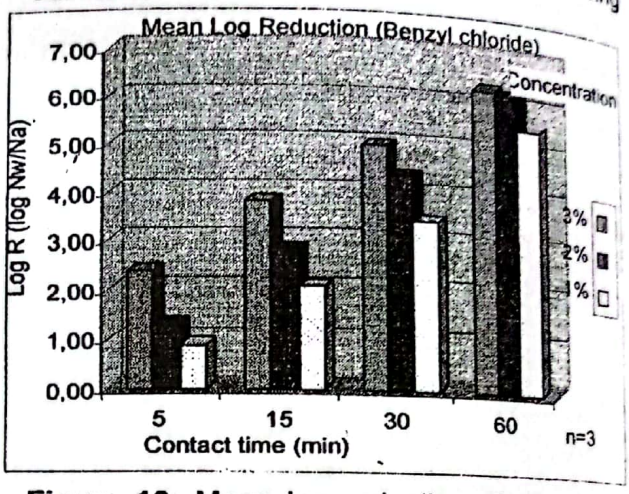

Figure 16: Mean log reduction of *Escherichia coli* with benzyl-alkyl-dimethyl ammonium chloride in the surface test. Interfering substance 1% skim milk.

The required reduction of Pseudomonas aeruginosa in both suspension and surface tests was achieved with 1 % in 60 min contact time (Figure 15).

DISCUSSION

The production of safe, wholesome milk is the major concern of the dairy industry and this can be obtained through risk assessment which encompasses identifying the hazards that may affect the quality or safety of the milk or dairy products and controlling them at all stages of the process such that their risk to product contamination is minimised. In the dairy industries this is commonly referred to as Hazard Analysis Critical Control Point (HACCP-concept). Such hazards are usually described as, biological (bacteria, yeast, moulds, insects, pests and dust), chemical (cleaning chemicals), and physical (heat and pressure). A hazard analysis should be undertaken at earliest opportunity in the process of milk production via application of hygienic practices

which are usually referred to cleaning and disinfection or sanitation programmes. This process has three key advantages; ensures that milk products is not held up with in the equipment where it could deteriorate and affect product quality, prevents the contamination of the product with substances that would adversely affect the health of the consumer and reduction of the cleaning time of the equipment so lead to long life time of the equipment, reduction of the costs and opportunity for increased production (Technical Manual 377A/00, 2000).

Chemical disinfectants are those agents which are used primarily to destroy microorganisms and not merely to arrest their growth so used in dairy industries for any organisms whose continued existence would result in undesirable consequences. Chemical disinfectants should be used only when there are no other suitable means of control of harmful microorganisms and in practical no other physical or no other biocide-free alternatives. They should be authorized and registered before marketing to ensure that when properly used for the purpose intended they are sufficiently effective, have no unacceptable effect on their target species, donít cause undesirable resistance and no harmful effects on human or animal health and on the environment (Bˆhm, 2002; Bessems, 2003).

Since a few years there are DIN EN standards available to test efficacy of disinfectant products which are intended to be used in the milk industries. In Germany previous DVG guidelines (2000) where used to test disinfectants for intended use in the food industries using a quantitative suspension test for fixing the recommended use concentrations. The advantage of this test methods was that they took into account the practical conditions in the field of application, but had the disadvantage that they didnít include the DIN EN methods. Since a few months there are new DVG guidelines for testing of disinfectant efficacy in the food industries available which are based on and include the method of EN standards but also keep the advantages of the former DVG-testing procedure. One main disadvantage of the EN-Standards is that there are no reference substances named. To have the possibility to compare the efficacy of disinfectants on the market and to assess i.e. changes in the susceptibility of test organisms over time (internal control) it is essential to use reference substances parallel in testing procedure. To fill in this blank the DVG included reference substances in the recently published guidelines which have to be tested in parallel to the actual test. Up to now there are only few data available, but it is necessary to have a broad data base for an impartial validation of disinfectant testing expertises and for a comparison of products available on the market, too.

Another advantage of the DVG-guidelines is the implementation of the MPN method for the deter-

mination the number of surviving test organisms. This technique is easy to perform and cost effective. The observation of growth was more accurate in the broth than on solid media, the pattern of growth can be observed visually, and the incubation period can be prolonged if necessary. Another point is that the neutralizer is added to each broth tube so transferred residual of the disinfectant is neutralized over a longer contact time and the sublethal damaged bacteria had a chance of being resuscitated. As mentioned before a further advantage of the MPN technique is that the incubation time in the broth tubes is not limited as it is on solid media where the tubes can be incubated more than three days without problems of media dryness during incubation and the bacteria could grow in broth without dependency on growth space. These observations correspond to the findings of i.e. DE Man (1983); Black (1996); Kamp et al. (2003) and Hunsinger et al. (2005). They also state that the MPN method is especially useful in situations where there is an advantage using broth over solid medium because many organisms are not good forming colonies, such as highly motile organisms or those organisms with quick growth or big colony size. Also, when sample contain too few organisms to give reliable measures of population size the MPN is used because one single colony inoculated in broth medium could show growth and the presence is easier to be observed from turbidity of broth tube. More than these advantages the possibility of contamination is smaller and the technique is quite easy to perform so MPN method offers an economic way for time and effort (DE Man, 1983; Black 1996). Furthermore the MPN method offers a possibility to minimize the broth medium volume required by using a minititter method which basically is a mini form of a normal MPN method. The advantages of this technique are reduction of working time and material as arising of trial quantity and reduction of substance doses needed for testing. This method also recommended by Kleiner and Trenner (1988) for colony counting in quantitative disinfectant testing.

Suspension test has a number of benefits as it is relatively simple and donít require specialized or expensive laboratory equipment and other than labor costs is cheaper to perform (Reybrouck 1992; Bloomfield et al., 1994). It is also well defined and is thus, within normal microbiological limits, repeatable and reproducible. Within this methodology it is also possible to test a wide range of variables including contact time, temperature, microorganism type and interfering substances (Holah et al., 1998). The major limitation of suspension test, however, is that it doesnít necessarily reflect in-use conditions (Holah et al. 1998), it also canít exclude ineffective disinfectants because it is too non-specific (Reybrouck 1991). The results of suspension tests therefore generally should not be used to fix use recommendations-with the exception of use recommen-

dations for Cleaning In Place (CIP) procedures. So, the main test for disinfectants efficacy on surfaces is the surface test which should cover the real life conditions found in dairies and in milk industries and subsequently lead to use recommendation for the practical applications (Spicher and Peters, 1997) .

This study deals with four chemical disinfectants, which represent products which are directly used in the milk industries, and are used as reference substances for main disinfectant compounds in the DVG-guidelines (2007). It is necessary to choose one reference substance for each product group, because only then it is possible to compare activity of products on the market-otherwise you only could use the results of the reference substances to check sensitivity of the test organisms (B^hm, 2002; Bessems, 2003). All these groups are used in the same time in dairies and dairy industries for disinfection of tanks, containers, filters, mixing machine, pipelines, bottle washing (rinse water), bottles centrifuges, pasteurizer, evaporator, general plant cleaning, air sanitation by fogging (bottling hall) and environmental hygiene. The test organisms were *Staphylococcus aureus* (ATCC 6538) and Enterococcus hirae (ATCC 10541) which selected in the performace of the most common Gram positive microorganisms in dairies and *Pseudomonas aeruginosa* (ATCC 15442) and Escherichia coli (ATCC 10536) which are far more resistant to disinfectants and also a significant Gram negative pathogens (Holah et al., 1990).

From the previously mentioned results it is achieved that when we used suspension test for determining the bactericidal efficacy of the tested reference disinfectants against tested organisms, *Staphylococcus aureus* (ATCC 6538) and Pseudomonas aeruginosa (ATCC 15442) were highly sensitive to formic acid while, *Escherichia coli* (ATCC 10536) and *Enterococcus hirae* (ATCC 10541) were more resistant. With application of peracetic acid the most resistant microorganisms were *Staphylococcus aureus* and *Escherichia coli.* While, the other two bacterial strains were highly suscepitible. With glutaraldehyde the highly sensitive microorgamnisms were Enterococcus hirae and because they need shorter contact time than the other tested microorganisms. Benzyl-alkyl-dimethyl ammonium chloride showed higher bactericidal effect against *Enterococcus hirae* and *Pseudomonas aeruginosa* than against *Staphylococcus aureus* and Escherichia coli which needed longer exposure times at the same concentration.

Generally the contact times and/or concentrations necessary to inactivate the test organisms were higher in the surface test than in the suspension test. Especially the Gram-negative test organisms showed higher resistance against the tested reference substances. Higher resistance of Gram-

negative bacteria has also been reported by Nikaido and Vaara (1985) and it was attributed to the presence of lipopolysaccharides in their outer membrane which making it naturally resistant to antibacterial agents. Although, we noticed that Gram negative test organisms were affected by prolonged drying time, when the test suspension was dried longer than 55 min on the steel surfaces, while Gram positive organisms were not affected. These results correspond to some studies which stated that Gram negative test organisms are reduced during the drying time on the steel surface. Reybrouck (1975) mentioned that the longer the drying time the higher is the problem that Gram negative test organisms are reduced. Also, Höller and Gundermann (1990) reported the same result especially with Pseudomonas aeruginosa. Bloomfield et al. (1994); Van Klingeren (1995) and Hunsinger (2005) confirmed those findings. One limiting factor in this context seems to be the interfering substance added. When interfering substance is added to the test the dying rate of Gram negative test organisms during drying time is reduced, these findingens are in agreement with results of Hirai (1991) and Abele (2004).

The choice of interfering substance and also the choice of the carrier material are factors influencing the test results (Haneke, 1991; Spicher and Peters, 1998; Bremer, 2003; Hunsinger et al., 2005 and Tilgner, 2007). This is obvious when comparing the results of steel carrier test in the actual study with previous work which has been done by i.e. Hunsinger (2005) we found that shorter exposure times were necessary at the same concentrations when using glutaraldehyde, formic acid and peracetic acid. This may be attributed to the use of highly concentrated interfering substance (yeast extract 10g/L + bovine albumin 10g/L) which was used in the study of Hunsinger (2005). The interfering substance used in this study was skim milk because milk is the soiling condition in dairies and dairy industries. Also, to make test condition nearly similar to that what happened naturally in the field of trial (standardised test procedure close to real life conditions) where the efficacy of disinfectants had been reduced in the presence of soiling materials even small quantities as a result of the reaction with organic matter and subsequently reduce the microbicidal effect of disinfectants (Böhm, 2002). Besides the reaction of the interfering substance with the disinfectant skim milk appears to have protective properties to test organisms against drying effect mainly with Gram negative organisms. This appears when using formic acid as reference substance against Gram negative test organisms because we need higher concentrations in the same contact time. Also, with peracetic acid and benzyl-alkyl-dimethyl ammonium chloride applications higher concentrations respectively prolonged exposure time were required. This also was observed with Gram positive test

organisms when using peracetic acid as reference substance.

From this study it is evident that disinfection of dairies either dairy farm or dairy plant is important for both public health and economic via avoidance of microbial contamination of milk from animals or from dairy utensils and equipment or other contact surfaces. The importance of applying suspension test and surface test together is that one should be aware of the specific advantages and shortcomings of every test, further a test must be seen as a part of a complete testing scheme and the predicting value of one test in itself is relatively low (Reybrouck, 1998). Also, use recommendations for disinfectants intended to be used in the milk industries canít be based on suspension test results. This test only applies as screening test to evaluate i.e. the influence of interfering substance and temperature on disinfectant efficacy. It is important, to be aware that surface attached bacteria are more resistant to biocides, especially when they are dried to the surface together with an interfering substance as they are in the used test method. Disinfectant tests don't take into account the level of microbial stress resulting from the cleaning action but only indicate that a disinfectant has antimicrobial properties in suspension or on surfaces and don't necessarily reflect its activity in practices (temperature, other factors influencing activity). Surface tests with test conditions as near to practicle condition as possible therefore should be used to determine in use concentrations and reference substances should be tested in parallel to the actual test to verify the stability of the test organisms and give guide to compare disinfectant products available on the market.

CONCLUSION

From this study we concluded that it is a good approach to include reference substances in the DVG- testing guidelines (2007). This is essential to check the test organisms for stability/ resistance. It is also necessary to have several different reference substance for each possible disinfectant compound to compare product efficacy. The reference substances chosen according the DVG-guidelines are able to cover disinfectant compounds normally used not only in the food industries especially milk industry and therefore, they should be generally used as reference substances. Another advantage is the use of the MPN method for determination of viable counts, because this can be easily performed and offeres an economic way for time, effort and costs-where less effort is needed for the testing procedure as well as for counting of the colonies.

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تعيين كفاءة المطهرات الكيميائية في القضاء علي بعض الميكروبات الموجبة والسالبة جرام في معامل الألبان تحت ظروف قياسية (الجمعية الطبية البيطرية الألمانية لتقيم كفاءة المطهرات الكيميائية)

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تم دراسة كفاءة أربعة مطهرات قياسية مدونة بدليل الجمعية الطبية البيطرية الألمانية لتقيم كفاءة المطهرات الكيميائية للقضاء علي الميكروبات الموجبة والسالبة جرام في معامل الألبان في وجود المواد العضوية (اللبن). وقد أسفرت النتائج علي أنه في حالة استخدام اختبار المحلول المعلق كانت ميكروبات المكور العنقودي الذهبي و الزوائف من نوع اريجينوزا أكثر حساسية لحامض الفورميك عند استخدامه كمادة قياسية. أما مع حامض البيراسيتك كانت اكثر الميكروبات مقاومة هي ميكروب المكور العنقودي الذهبي و ميكروب الايشريشياكولاي. في حين أن ميكروبات المكور المعوي من نوع هيرى و ميكروب الايشريشياكولاي كانت الأكثر حساسية للجلوتارألدهيد. أما البنزيل الكيل داي ميثيل أمونيوم كلورايد كان أكثر تأثيرا علي ميكروبات الايشريشياكولاي و الزوائف من نوع اريجينوزا. كما أوضحت النتائج أيضا أن زيادة تركيز المطهرات الكيميائية و إطالة فترة التعرض مهمة جدا في حالة جفاف الميكروبات علي الأسطح المعدنية بالمقارنة عند وجود نفس الميكروبات في محلول معلق خاصة مع الميكروبات السالبة جرام وكان ذلك واضحا عند استخدام حامض الفورميك كمادة قياسية حيث كان هناك زيادة في التركيز مع نفس زمن التعرض أما مع حامض البيراسيتك و البنزيل الكيل داي ميثيل أمونيوم كلورايد كان هناك زيادة في التركيز بالتوالي مع زمن تعرض أطول. في حين أن الميكروبات الموجبة جرام أعطت هذه النتائج عند استخدام حامض البيراسيتك كمادة قياسية. كما أوضحت النتائج وجود فروق بين الأربعة ميكروبات المختبرة حيث كان ميكروب الايشريشياكولاي أكثر مقاومة لحامض الفورميك. أما الزوائف من نوع اريجينوزا كانت أكثر مقاومة لحامض البيراسيتك. بينما أعطي الجلوتارألدهيد نفس النتائج مع جميع الميكروبات المختبرة. وكان المكور المعوي من نوع هيرى أكثر حساسية للبنزيل الكيل داي ميثيل أمونيوم كلورايد. وهذه الحقائق تؤكد علي ضرورة إتباع الدقة في اختيار المطهر الكيميائي المناسب للأسطح الملوثة في مزارع ومصانع اللبان خصوصا في وجود المواد العضوية كما توجب ضرورة استخدام مطهرات قياسية لمقارنة كفاءة المطهرات الكيميائية في تأثيرها علي الميكروبات المختلفة.