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Note

Bactericidal Activities of a Wet Disinfectant Mat in a Food Factory

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The antibacterial activity of the agent of a wet disinfectant mat (WDM) utilizing benzalkonium chloride (BAC) and sodium carbonate was researched. The agent of the WDM showed a bactericidal activity in which there was a decrease of 10^7 cfu/ml or more in all the strains tested in an exposure of 30 s or more. On the WDM, *Staphylococcus aureus* showed blebs after 30 min, and remarkable destruction of bacterial body was also observed. After 2.5 h on the WDM, the whole bacterial body of *Salmonella enteritidis* was covered with many irregular blebs. In the early stage at 5 min after pouring the bacterial suspension on the WDM, embedding of *S. enteritidis* in the drug layer was observed. These results suggested that the WDM would trap and kill microorganisms in food factories.

Key words : Wetting disinfecting mat/Benzalkonium chloride/Sodium carbonate/Bactericidal activity /Morphological change.

To prevent food poisoning, it is important to identify the source of contamination and block the route of infection. It is known that in places where factory workers often come and go, the levels of airborne bacteria are markedly higher (Sugano et al., 1990). There is a risk that food materials will not only be directly contaminated by these airborne bacteria but also indirectly by workers, fingers contaminated by these same airborne bacteria. Therefore, maintaining the cleanliness of the working place is very important. In the examination of the distribution of contaminating bacteria in the working place, contamination is known to be concentrated on the floor and horizontal surfaces rather than on the wall and vertical surfaces (Ishigaki et al., 1991 ; Komemushi et al., 1991 ; Nakahama et al., 1991). These bacteria attached to the floor surface are transferred to other floor areas by means of footwear (Watabe et al., 1994), and are concentrated on the path lines of worker traffic (Ogiri et al., 1990). In addition, numbers of bacteria on the floor are correlated with those of airborne bacteria (Ogase, 1996). Therefore, paying attention to the fact that bacteria on a floor are transferred to other floor areas via footwear, we made a WDM for the purpose of removing and killing bacteria attached to the soles of the footwear of workers, especially in food factories. As the agent of WDM, BAC and sodium carbonate were used concomitantly. BAC is commonly used in food facilities and medical institutions, and its bactericidal activity and its effects on bacterial morphology have been reported (Jono and Higashide, 1987 ; Kourai, 1997). However, the bactericidal activity of the combined use of BAC and sodium carbonate is little known, except for the report of Takahashi et al. (1989), and morphological change of microorganisms trapped on the mat has not been reported yet. In this study, we investigated the bactericidal activity

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resulting from the combined use of BAC and sodium carbonate, and observed morphological changes of microorganisms trapped by the WDM using a scanning electron microscope (SEM).

The WDM was wet with a mixed solution of BAC and sodium carbonate, and dried. It was moistened with 300 ml of water per WDM just before use. The concentrations of BAC and sodium carbonate in the moistened state were adjusted to be 1.0% (v/v) and 0.5% (w/v) respectively. The pH at that time was 10.5. *Bacillus subtilis* IFO 12113, *Clostridium botulinum* type A 62, *Clostridium perfringens* ATCC 3624, *Staphylococcus aureus* FDA209P, *Escherichia coli* IFO 3301, *Escherichia coli* ATCC 43888 O157:H7 (vero toxin non productive strain), *Salmonella enteritidis* IFO 3313 and *Vibrio parahaemolyticus* Na 2 (Nakasone and Iwanaga, 1991) were used in this study. The bacteria were grown in heart infusion broth (Nissui Seiyaku Co.) at 37 °C for 18h, harvested from the media and washed twice with CMF- Dulbecco's PBS (PBS) (Dulbecco and Vogt, 1954) by centrifugation. Finally, bacterial cells were resuspended in PBS. *V. parahaemolyticus* was grown in nutrient broth supplemented with 3% (w/v) NaCl (Nakasone and Iwanaga, 1991). The bacteria was washed and suspended with 3% (w/v) NaCl in water. *C. botulinum* and *C. perfringens* were grown in a GAM broth (Nissui Pharmaceutical Co.) at 35 °C for 18 - 20 h anaerobically.

Testing of antibacterial activity was done according to the modified JIS 1902 (Japanese Industrial Standards, 1998). The bacterial suspension of each of test strain was diluted with PBS or 3% (w/v) NaCl in water to 10^5 to 10^6 cfu/ml. Each (1 ml) of these bacterial suspensions was mixed with dissolved heart infusion agar medium (Eiken Chemical Co.) after being cooled to 45 to 50 °C, and the mixture was poured into a sterilized plate (ϕ 90 mm). *V. parahaemolyticus* was mixed with dissolved nutrient agar medium supplemented with 3% (w/v) NaCl. *C. botulinum* and *C. perfringens* were spread on a GAM agar plate (Nissui Pharmaceutical Co.). Test patches were prepared by cutting the WDM into disks, 20 mm in diameter. The surface of each test patch was moistened with 0.21 ml of sterilized distilled water. After being moistened, the WDM patch was placed on each plate in contact with the surface, and incubated at 35 °C for 48 h. After culturing, the diameter of the clear layer that represents the growth inhibition of bacterial strains were measured. Antibacterial activity was calculated using the following equation: [Index of antibacterial activity = (diameter of inhibition zone including the test patch - diameter of test patch) ÷ 2]

Among the bacterial strains used in the antibacterial test, *E. coli, S. aureus, S. enteritidis* and *V. parahaemolyticus* were used in the examination of the bactericidal activity of the agent of WDM. This agent was prepared at two-fold concentration of the agent of WDM , and the agent (12 ml) was diluted with an equal volume of PBS or 3% (w/v) NaCl in water. To this solution, 1 ml of bacterial suspension containing 10^{9-10} cfu/ml bacteria was added. Just after addition, stirring (250 rpm) was started at 20 to 25 °C and 0.2 ml of the suspension was sampled at 30 s, 1 min, 2 min, 5 min and 30 min later. Each sample underwent 10-fold stepwise dilution with Soy Bean Casein Digest Broth with Lecithin and Polysorbate (SCDLP) to neutralize the bactericidal activity of the agent of WDM, and 0.1 ml of the dilution was spread on a SCDLP solid medium (Eiken Chemical Co.). The medium was incubated at 35 °C for 48 h, and then viable cell colonies were counted. For *V. parahaemolyticus*, 10-fold stepwise dilution was made with 3% (w/v) NaCl in water, spread on nutrient agar medium supplemented with 3% (w/v) NaCl and incubated similarly. A two-fold dilution of the agent of WDM was prepared, and the bactericidal test was performed under the same conditions for comparison between the two-fold and original concentrations of the agent.

For observation of microstructural changes of bacteria on the WDM, a *S. aureus* or *S. enteritidis* suspension (2 ml of 10^{7-8} cfu/ml) was applied to a WDM patch (5 cm^2). After dispersion of bacterial suspension in the WDM patch, the patch was incubated at 20 to 25 °C for 5 min, 30 min or 2.5 h. For observation of structural changes of bacteria in the agent of WDM (in a test tube), 1 ml of about 10^9 cfu/ml bacterial suspension was added to 24 ml of the agent of WDM, and the mixture was stirred (250 rpm) at 20 to 25 °C for 5 min, 30 min or 2.5 h. After treatment, the bacterial cells were washed twice with PBS by centrifugation, and resuspended in PBS. Each (100 µl) of these bacterial suspensions was dropped on a cell disk R1 (ϕ 13.5mm, Sumitomo bakelite Co.) that had been coated with 0.1% (w/v) poly-L-lysine hydrobromide (Wako Pure Chemical Industries, Ltd.) aqueous solution, and attached. Bacterial suspensions that were not treated with the agent of WDM underwent PBS washing once, were resuspended at 10^{5-6} cfu/ml, and were attached to the cell disk. These samples were fixed with glutaraldehyde (2%, v/v) at room temperature for 1 h, dehydrated with ethanol and dried in a CO_2 atmosphere with a critical-point dryer (Hitachi HCP-2). The samples were coated with Pt-Pd using an ion sputter (E-1030, Hitachi), and observed by using the SEM (Hitachi S-900, acceleration voltage of 6kV).

Different from the conventional adhesive mat for

trapping dust, the WDM used in the present study not only had dust-trapping property but also exhibited immediate and long-lasting bactericidal activity. As the agent of WDM, BAC and sodium carbonate were used in combination. Although enhancement of bactericidal activity and effective killing of disinfectant-resistant bacteria by the combined use of BAC with sodium carbonate are known, only a few related reports are available (Jono and Higashide, 1986 ; Yamazaki et al., 1990). The antibacterial activity of the agent of WDM was observed by means of the formation of a growth inhibition diameter. Among the Gram-positive bacilli among strains used in the test, *C. botulinum* showed the largest diameter of 15.6 mm. *C. perfringens* showed a relatively small inhibition diameter of 8.5 mm. *B. subtilis* showed a slightly large diameter of 13.3 mm, and a Gram-positive coccus, *S. aureus*, gave an inhibition diameter of 9.8 mm. Growth-inhibition diameters of Gram-negative bacilli, *E. coli* IFO 3301, *E. coli* ATCC 43888 O157: H7 (vero toxin non productive strain), *S. enteritidis* and *V. parahaemolyticus*, were 5.7 mm, 6.7 mm, 6.0 mm and 6.2 mm, respectively. Each was less than 7 mm, and no difference among these strains was observed. These results were in line with the general tendency that the bactericidal activity of BAC against Gram-positive bacteria is stronger than that against Gram-negative bacteria (Miwatani, 1991; Nishihara, 1998). Among these bacterial strains, a total of 4 species, *E. coli* which is used as an index in the food sanitation test, and *S. aureus*, *S. enteritidis* and *V. parahaemolyticus* which are causative bacteria in food poisoning cases, were selected, and the bactericidal activity of the agent of WDM against them was examined.

For determination of the treating time with the agent of WDM, we counted the number of persons who passed though a few points in a food manufacturing facility with 46 persons during 8 h of work, and found that a total of 400 to 700 persons passed though each point. Therefore, the time interval between passages was 0.68 to 1.2 min. This data seemed to indicate that contaminant bacteria carried in by one individual on the bottom surfaces of his footwear should be killed within about 1 min on the mat. If this condition is not satisfied, contaminant bacteria will accumulate and may proliferate on the mat, and in consequence, the mat itself may become a source of contamination similarly to the case of the adhesive mat (Fukada et al., 1987). Although the measuring time of the bactericidal activity of the agent of WDM in this study was set at a total of 5 points, at 30s, 1min, 2min, 5min and 30min of exposure, we thought that great importance should be placed on the 30s data because of the

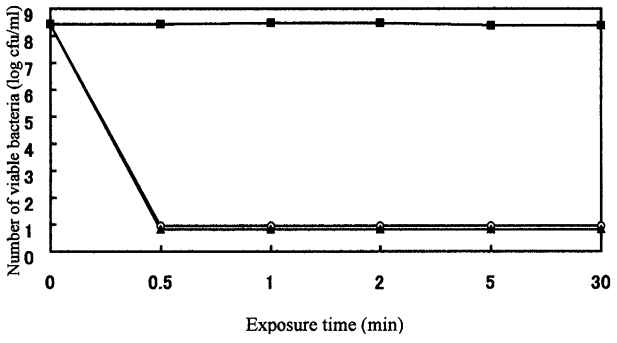

FIG. 1. Bactericidal effect of the agent of WDM on *E. coli*. Symbols: ■, no treatment; ▲, treatment with the agent of WDM; ○, treatment with a 2-fold dilution of the agent of WDM.

above reason. When treated with the agent of WDM for 30 s, *E. coli* decreased more than 10^7 cfu/ml (Fig. 1). A similar test was performed using a two-fold dilution of the agent of WDM, and results similar to those obtained with the agent of WDM were obtained. The results obtained with *S. aureus, S. enteritidis* and *V. parahaemolyticus* were similar to those obtained with *E. coli*, and a decrease by more than 10^7 cfu/ml was observed after treatment for 30 s. The bactericidal activity of a two-fold dilution of the agent of WDM was similar to that of the WDM agent. The bactericidal activity of the agent of WDM was marked, and reduction by 10^7 cfu/ml or more was found in all the tested bacterial strains. The purpose of WDM is to trap and kill bacteria attached to the soles of footwear. We counted the number of bacteria attached to the soles of the footwear of 10 subjects and found 10^2 to 10^3 cfu/foot. Therefore, the bactericidal activity of the WDM in this study appeared to be effective.

Changes in microstructure of bacteria on the WDM were observed with a SEM. *S. aureus* bodies that were not treated with the agent of WDM are shown in Fig. 2A. The bacterial bodies in the normal state showed a smooth surface layer. After 5 min of treatment with the agent of WDM, cracks were found in the surface layer (Fig. 2B, arrowhead). In addition, many large and small blebs were found on the bacterial body surface (Fig. 2B, arrow). Depressions were found on a few of these blebs (Fig. 2B, long arrow). Bacterial bodies on the WDM are shown in Figs. 2C and 2D. After 30 min, bacterial bodies embedded in the drug layer were found (Fig. 2C, arrow). An enlargement of Fig. 2C is shown in Fig. 2D. On the WDM, cracks (Fig. 2D, arrowhead) and blebs (Fig. 2D, arrow) similar to those observed after treatment with the agent of WDM were found, indicating severe destruction of bacterial bodies (Fig. 2D).

S. enteritidis bodies that were not treated with the

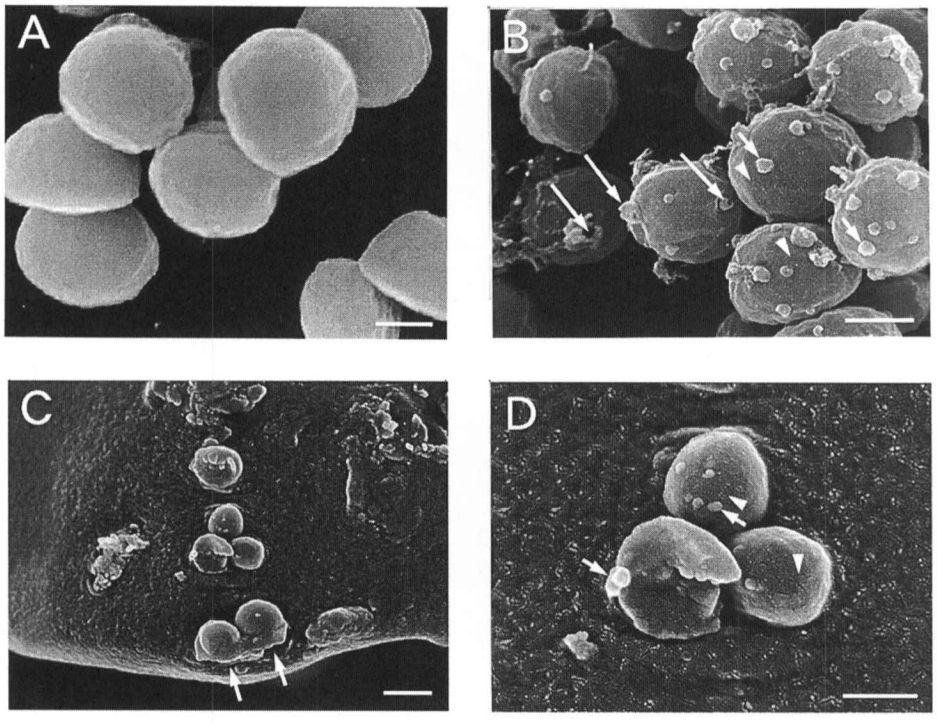

FIG. 2. Electron micrographs of *S. aureus*. (A) Bacterial bodies untreated with the agent of WDM. Scale bar, 0.5 μm. (B) Bacterial bodies treated with the agent of WDM for 5 min. Scale bar, 0.5 μm. (C) Bacterial suspension was poured on and exposed to the WDM for 30 min. Scale bar, 1 μm. (D) Magnification of Fig. 2C. Scale bar, 0.5 μm.

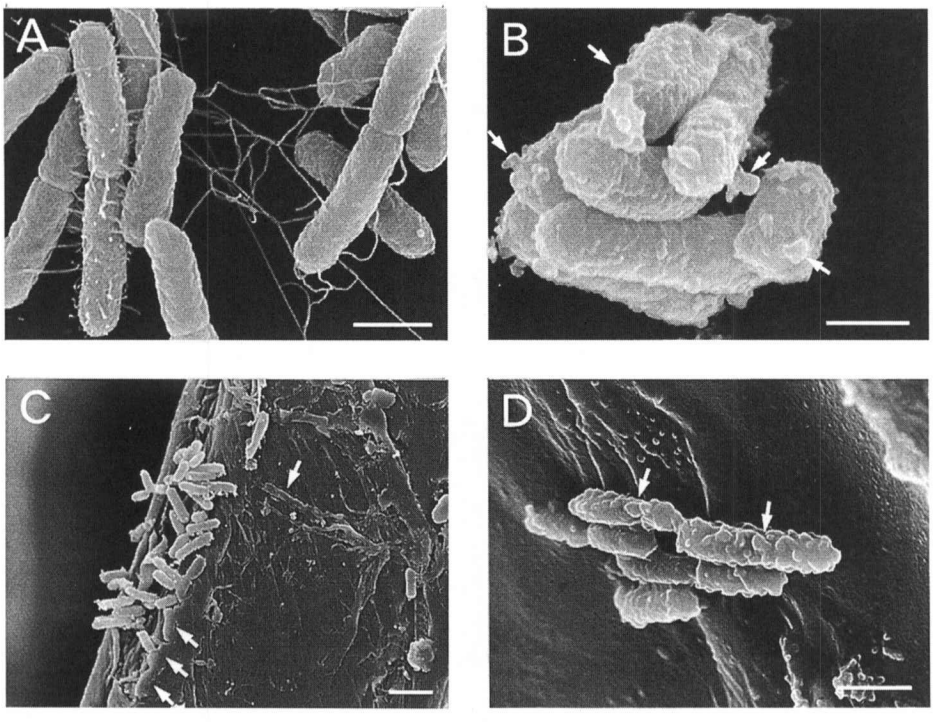

FIG. 3. Electron micrographs of *S. enteritidis*. (A) Bacterial bodies untreated with the agent of WDM. Scale bar, 1 μm. (B) Bacterial bodies treatment with the WDM agent for 2.5 h. Scale bar, 0.5 μm. (C) Bacterial suspension was poured on and exposed to the WDM for 5min. Scale bar, 2 μm. (D) Bacterial suspension was poured on and exposed to the WDM for 2.5 h. Scale bar, 1 μm.

agent of WDM are shown in Fig. 3A. A flagella extending from the body surface and surrounding the body was found on the normal cell body (Fig. 3A). After 5 min of treatment with the agent of WDM, blebs developed (data not shown), and after 2.5 h, many irregular blebs were observed (Fig. 3B, arrow).

Distinct morphological changes were found after treatment with the agent of WDM for 5 min in *S. aureus*, and 2.5 h in *S. enteritidis*. This time difference between these species seemed to be attributed to the difference between Gram-positive and Gram-negative bacteria in their permeability to drugs due to the structural differences in their cell walls. These blebs were similar to the changes caused on the cell-surface of *E. coli* by a quaternary ammonium salt reported by Kourai (1997) : they reported that the action of a quaternary ammonium salt on bacteria was quick and the surface structure of bacteria was destroyed in a very short period, and that the mechanism of action was injury to the cell membrane and cell wall (Kourai, 1997). In our present study, since the time of exposure was 5 min and short, and depressions were found on some of blebs, the mechanism of action appears to be similar to that of the quaternary ammonium salt, but further examination is necessary.

Bacterial bodies poured on the WDM were embedded in the drug layer after 5 min (Fig. 3C, arrow). Bacterial morphological changes observed on the WDM were similar to those found after treatment with the agent of WDM, and the entire surface of bacterial body was covered with irregular blebs after 2.5 h (Fig. 3D, arrow). No surrounding flagella extending from the bacterial body was found on bacterial bodies treated with the agent of WDM or those on the WDM. In the present study, morphological observation was performed after 5 min, 30 min, and 2.5 h of placement on the WDM, at 3 points. A slightly longer time seemed to be necessary for bacteria placed on the WDM to show distinct morphological changes similar to those exposed directly to the agent of WDM. This appears to be related to the time required for the gradual elution of the drugs contained in the WDM after water is sprayed. However, in the early stage at 5 min after application, it was observed that *S. enteritidis* was buried in the drug layer on the WDM. These observations seemed to indicate a high probability that bacteria once captured by the WDM will be killed there. In the examination of microstructure of bacterial bodies trapped in the WDM, marked destruction of cells was found in both species examined, suggesting possible bactericidal activity on these bacterial cells. From these results, the WDM used in this study appears capable of killing trapped microorganisms in food factories.

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