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Surfactant-disinfectant resistance of *Salmonella* and *Staphylococcus* adhered and dried on surfaces with egg compounds

Takashi Kuda [a,*], Taichiro Iwase [a], Chaturongkasmurit Yuphakhun [a], Hajime Takahashi [a], Takashi Koyanagi [b], Bon Kimura [a]

[a] Department of Food Science and Technology, Tokyo University of Marine Science and Technology, Konan, Minato-ku, Tokyo 108-8477, Japan
[b] Department of Food Science, Ishikawa Prefectural University, Suematsu, Ishikawa 921-8836, Japan

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ABSTRACT

To confirm the importance of eliminating food sediments from the surfaces of food-related environments, we examined the resistance of pathogenic bacteria (*Salmonella* Typhimurium and *Staphylococcus aureus*) cells, dried and adhered on glass with 25% w/v egg albumen, 25% yolk or 50% whole egg solutions, against benzalkonium chloride and alkyldiaminoethylglycine hydrochloride. Bacterial suspensions (0.1 ml of 8 log cfu/ml) were put on 47 mmφ glass dishes and dried at room temperature (20–24 °C) for 180 min in a bio safety cabinet with ventilation. Although the viable cells in distilled water decreased 2.0 (*S. aureus*)–3.5 (*S.* Typhimurium) log fold during the drying period, the egg compounds protected the bacteria. The disinfectant treatments (2.0 mg/ml for 10 min) showed a clear bactericidal effect in the absence of egg compounds. However, the bactericidal effect disappeared in the presence of yolk and whole egg. Imaging before and after drying and the disinfectant treatments were carried out using a phase-contrast microscope and an atomic force microscope. The protective effect of egg compounds on bacterial viability disappeared with a proper washing process.

1. Introduction

Salmonella is a well-documented pathogen known to occur in a wide range of foods, especially eggs and egg products (Rivoal et al., 2009). Most cases of human infection with *Salmonella enterica* subsp. *enterica* serotype Enteritidis result from the consumption of contaminated raw eggs (Mead et al., 1999). Furthermore, *salmonellosis* from *S.* Typhimurium has remained relatively stable or increased in Europe, the US and the other countries (Weill and Grimont, 2005; Jamshidi et al., 2010). *Staphylococcus aureus* is also a leading cause of gastroenteritis resulting from the consumption of contaminated foods. Staphylococcal food poisoning is due to the absorption of staphylococcal enterotoxins preformed in various foods, including eggs and egg products (Haeghebaert et al., 2002; Loir et al., 2003).

Adhesion of microorganisms to equipment surfaces has the potential to transmit pathogens to food, and this is apparent in the food-processing industry (Giauris and Nychas, 2006; Simões et al., 2010) and in the domestic environment (Humphrey et al., 2001; Hayson and Sharp, 2005). It is known that microorganisms on the inner and/or wet surfaces of food and medical utensils and equipment often form biofilms that exhibit tolerance to various stresses (McNeill and Hamilton, 2004; Kubota et al., 2009). In particular, there are many reports about the biofilms of *Pseudomonas aeruginosa* (Parsek and Tolker-Nielsen, 2008), *S. aureus* (Kwon et al., 2008), *Listeria monocytogenes* (Takahashi et al., 2010) and *S.* Typhimurium (Zaklkhany et al., 2010), due to their strong resistance against disinfectants and their medical importance in disease, such as nosocomial infections.

There are many kinds of disinfectants for food utensils, such as alcoholic and hypochloric solutions. Quaternary ammonium compounds and amphoteric surfactants are commonly used as disinfectants in food production environments (Maillard, 2002). Benzalkonium chloride (BAC) is a quaternary ammonium compound that is widely used for sanitation in food-processing environments. BAC acts on general membrane permeability, causing the cytolytic leakage of cytoplasmic materials at low concentrations. Alkyldiaminoethylglycine hydrochloride (AGH) is an amphoteric surfactant used for sanitation (Sakagami and Kajimura, 2002). It is known that amphoteric surfactants cause leakage of intracellular components in bacteria (Maillard, 2002).

On the other hand, it is considered that food elements protect microbial cells when the adhered cells are dried on the surfaces of food utensils and equipment (Leslie et al., 1995). Although there are many reports about the effect of dirty conditions on bacterial

* Corresponding author. Tel./fax: +81 35 463 0602.
E-mail address: kuda@kaiyodai.ac.jp (T. Kuda).

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viability determined by the European Standard EN-1276 (1997), these studies were carried out in suspensions in tubes (Briñez et al., 2006; Simões et al., 2010) that maybe different from the dried surface environment. We previously reported that a standard concentration (2 mg/ml) of BAC did not decrease the number of bacterial cells dried on a stainless steel surface with milk (Kuda et al., 2008). The protective efficiency of milk on bacteria disappeared if washed with a neutralized detergent. However, details of the efficiency of disinfectants on dried cells with or without food elements are not clear.

In this study, to determine the importance of proper washing and detachment of food sediments from the surfaces of food utensils before chemical disinfection, the protective effect of egg albumen, yolk and whole egg on *S.* Typhimurium and *S. aureus*, dried and adhered to a glass dish, was examined using conventional culture methods, phase-contrast microscopy and atomic force microscopy.

2. Material and methods

2.1. Bacterial cultures

S. enterica subsp. *enterica* serotype Typhimurium NBRC 13245 and *S. aureus* NBRC 12732 were employed to investigate attachment and disinfection treatments on utensil surfaces. To produce cultures, the bacterial cells were inoculated into 10 ml of Trypticase Soy Broth (TSB, Becton, Dickinson and Co., Sparks, MD) and incubated at 37 °C for 20 h. The culture reached stationary phase.

2.2. Chemicals and surfaces

Ten percent benzalkonium chloride (BAC) solution was purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). Ten percent alkyldiaminoethylglycine hydrochloride (AGH: Hygieel) solution was purchased from Maruishi Pharmaceutical Co. (Osaka, Japan). Alkyl ether sulfuric acid ester sodium (AES), a neutralized detergent, was purchased from Kao Co. (Tokyo, Japan). Fresh hen's eggs were purchased from a retail shop in Tokyo.

Fifty-millimeter φ sus304 stainless steel dishes, 47-mmφ glass dishes and gamma ray-irradiated 50-mmφ polystyrene dishes were purchased from As One Co. (Osaka, Japan). In advance, the stainless steel and glass dishes underwent ultrasonication twice for 15 min, brushing for 60 s and autoclaving at 121 °C for 15 min.

2.3. Adhesion of S. Typhimurium to the three surfaces

The bacterial cells were attached according to the method previously reported (Kuda et al., 2008), with slight modifications. Bacterial suspensions were prepared from the stationary phase with TSB cultures (10 ml). Bacterial cells were centrifuged at 2000× *g* for 10 min and re-suspended in phosphate-buffered saline (Nissui Pharmaceutical Co., Tokyo, Japan); this washing process was carried out twice. The cells were finally re-suspended in 10 ml of distilled water, or with 25% w/v albumen, 25% egg yolk or 50% whole egg solutions. The cell concentration was about 8 log cfu/ml. The bacterial suspension (0.1 ml) was placed in the center of the dish ($n = 3$) and dried for 180 min at room temperature (20–24 °C) in a bio safety cabinet (Class IIA, Airtech Japan Co., Tokyo, Japan) with ventilation. After drying, the adhered cells were scraped and thoroughly suspended for 60 s with 5 ml TSB using a cell scraper (TPP, Switzerland). Then, the detached cell suspension (0.1 ml) was immediately diluted with PBS and plated on Trypticase Soy Agar (TSA, Becton, Dickinson and Co.) and incubated at 37 °C for 24 h.

2.4. BAC and AGH treatment and enumeration of viable cells on glass dishes

To determine the bactericidal effect of the disinfectants, the dried and adhered *S.* Typhimurium and *S. aureus* cells on the glass surface, prepared as above, were covered with 0.1 ml of 2.0 mg/ml BAC or AGH solution, which is the highest recommended and indicated concentration by the makers. After 10 min at room temperature, 5 ml of TSB was added. Then, cell viability was determined as above.

2.5. Microscopic observation

Cells in combination with the egg compounds were dried on glass dishes and observed using light microscopy at low (×50) and high (×1000) magnification. The effect of the disinfectants on cell suspensions was observed under a phase-contrast microscope (ODEO-Quattro, Iponacology, Tokyo, Japan) (Kuda et al., 2009). The effects of the egg compounds and disinfectants on the dried and adhered cells on glass surface were analyzed using atomic force microscopy (SPM-1000, Shimadzu, Kyoto, Japan) with a contact mode probe (Kuda et al., 2010).

2.6. Effect of water washing on adhered cells prior to disinfectant treatment

To determine the effect of washing on adhered cells with egg compounds on glass surfaces (as above), water washing treatment was carried out immediately as previously reported (Kuda et al., 2008). This method was modeled on domestic and food service environments. The adhered cells were washed twice using sterilized cotton swabs for 60 s with 4 ml of water. Then, the viable adhered cells on the glass surfaces were treated with 4 ml of water or 0.28 mg/ml AES for 10 min. After rinsing with water (5 ml, twice), 2 mg/ml BAC or AGH (1 ml) was added and incubated for 10 min. Finally, the disinfectant-treated dishes were rinsed twice more with 5 ml of water. After each treatment, the viable adhered cells were counted in the same way as above.

2.7. Statistical analysis

The cell viability data was expressed as the mean and standard deviation of log cfu/dish ($n = 3$). Statistical analysis was performed using the software EXCEL Statistic 5.0 (Esumi Co., Ltd., Tokyo, Japan). One-way ANOVA was used to assess differences. Then, individual means were compared by Duncan's multiple-range test. Significant differences were accepted at $p < 0.05$.

3. Results and discussion

3.1. Effect of drying on bacterial cells on three surfaces

In our previous study, the sensitivity of logarithmic or early stationary growth phase cells to drying was higher than that of the stationary growth phase cells (Kuda et al., 2008). It has been reported that the sigma factors produced in the stationary phase lead to the generation of proteins that self protect against acidic conditions, dryness, low temperature and reactive-oxidants (Pohlmann-Dietze et al., 2000; Valle et al., 2007). Therefore, we used stationary phase bacterial cells in this study.

The numbers of viable *S.* Typhimurium cells before and after the drying on stainless steel, glass or polystyrene, with or without egg compounds, are summarized in Fig. 1A. During the 180 min ventilation at room temperature, the viable cells in water were thoroughly dried and decreased from 7.3 to ≤4 log cfu/dish on stainless