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Short communication

Effect of quantity of food residues on resistance to desiccation of food-related pathogens adhered to a stainless steel surface

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ABSTRACT

In order to study the effect of food residues on the survival of food-borne pathogens, *Salmonella* Typhimurium, *Staphylococcus aureus*, and *Listeria monocytogenes* were subjected to drying conditions in the presence of small amounts of food such as carrot juice, aqueous solution of *nori*, milk, and soy-milk. After drying for 2 h at room temperature in the absence of food residue, cell counts of *S.* Typhimurium, *S. aureus*, and *L. monocytogenes* decreased from 8 to 3, 6, and 5 log cfu/dish, respectively. Five milligrams of fresh carrot, 0.05 mg dried *nori*, and 100 nL milk or soy milk per 10 mm φ surface were sufficient to demonstrate a protective effect on the adhered pathogens, as confirmed by atomic force microscopy. Results from this study suggest that small sediments of food, not only protein rich but also carbohydrate rich, increase the resistance of surface-adherent bacteria to desiccation, rendering sanitization processes ineffective and encouraging cross contamination.

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1. Introduction

Microbial adhesion to surfaces is a potential route of transmission of pathogens 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). Microorganisms within and/or on wet surfaces of utensils and medical equipment often form a biofilm, which exhibits resistance to various kinds of stress conditions (Boles and Singh, 2008; Finn et al., 2013). In particular biofilms formed by *Pseudomonas aeruginosa* (Baird et al., 2012; Murphy et al., 2014), *Staphylococcus aureus* (Chen et al., 2012; Kuda et al., 2011), *Listeria monocytogenes* (Chaturongkasumrit et al., 2011; Lourenço et al., 2013), and *Salmonella* Typhimurium (Nguyen and Yuk, 2013; Kuda et al., 2012) serious threats because of their strong resistance to disinfectants and their role in nosocomial infections.

We have previously reported that when food poisoning agents such as *Salmonella* Typhimurium and *S. aureus* were dried and adhered onto stainless steel or glass surfaces in the presence of nutrient-rich food residue such as milk, meat, and egg, they showed resistance to desiccation, surfactant disinfectants such as benzalkonium chloride, as well as 254-nm ultraviolet (UV)-C irradiation (Kuda et al., 2008, 2011, 2012; Li et al., 2014). This indicated that protein-, lipid-, and/or carbohydrate-rich food residue could protect pathogens from stress conditions. The gram-positive rod *L. monocytogenes,* regarded as tolerant to desiccation, is similarly protected by food residue (Takahashi et al., 2011). These studies indicate that washing and rinsing prior to sterilization are essential to meet the strictly sterile requirements for food safety. However, the quantum of food residue that is sufficient to protect pathogens from stress such as desiccation, disinfectants, or UV-C remains unclear.

In this study, we investigated the quantity-dependent effects of milk, soy milk, carrot, and laver *nori* residue on survival rates of *Salmonella* Typhimurium, *S. aureus*, and *L. monocytogenes* adhered to and desiccated on a stainless steel surface. The cells adhered in the presence of food residue were observed using atomic force microscopy.

2. Materials and methods

2.1. Bacterial culture and food material

Salmonella enterica subsp. *enterica* serotype Typhimurium NBRC 13245, *S. aureus* NBRC 12732, and *L. monocytogenes* Scott A were adhered onto utensil surfaces. To produce cultures, the bacterial cells were inoculated into 10 mL trypticase soy broth (TSB; Becton, Dickinson and Co.; Sparks, MD) and incubated at 37 °C for 20 h. Under these conditions, the culture reached stationary phase (Kuda et al., 2013).

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Fresh carrot, sundried sheets of laver *Porphyra* sp. (called *nori* in Japanese), milk, and soy milk were purchased from a retail shop in Tokyo. The carrot was minced with an equal amount of distilled water (DW) for 60 s in a blender (16-Speed Blender; Osaka Chemical; Osaka, Japan) and shaken for 60 min at room temperature. The carrot extract was collected by centrifugation (2500 *g* for 10 min) and sterilized by filtration with a 0.22-μm-pore filter. The dried *nori* was milled in the blender and autoclaved (121 °C for 15 min) with 40 time volumes of DW. After centrifugation, the supernatant was used as *nori* extract. The carrot extract, *nori* extract, milk, and soy milk samples were diluted 10-fold in DW to obtain 10^{-4} (0–10,000-fold diluted) solutions.

2.2. Adhesion of food pathogens to surfaces

Fifty-millimeter-φ stainless steel dishes were purchased from As One Co. (Osaka, Japan) and used as the experimental surface (Kuda et al., 2008). Prior to use, in order to equalize the effect of the surface conditions on the survival cell count (Bohinc et al., 2014), the steel dishes were ultra-sonicated twice for 15 min, brushed for 60 s, and autoclaved at 121 °C for 15 min. The bacterial cells were placed in the dish and attached as previously reported (Kuda et al., 2011), with slight modifications. Briefly, bacterial cells in the TSB culture were washed by centrifugation at 2000 *g* for 10 min at 4 °C and re-suspended in phosphate-buffered saline (PBS; Nissui Pharmaceutical Co.; Tokyo, Japan); this process was repeated twice. The cells were finally re-suspended in 10 mL DW or the diluted food samples such that the final cell concentration was approximately 8–9 log cfu/mL.

A bacterial suspension (0.1 mL) was placed in about 10 mm-φ of the center of the dish ($n = 3$) and dried for 120 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 detached by rubbing for 60 s using a sterile cotton swab and re-suspended in 5 mL TSB (Nissui Pharmaceutical). The detached cell suspension (0.1 mL) was immediately diluted in PBS, spread on trypticase soy agar (TSA; Becton, Dickinson and Co.) and incubated at 37 °C for 24 h.

2.3. Microscopic observations

To observe the bacterial cells, 0.01 ml of the cell suspension, prepared as same as above, was adhered by drying on a cover slip and the cells were observed using an atomic force microscope (AFM; Naio AFM; Nanosurf AG; Liestal, Switzerland) in dynamic force mode.

2.4. Statistical analysis

Bacterial cell viability was expressed in terms of mean and standard deviation of log cfu/dish ($n = 3$). Statistical analysis was performed using EXCEL Statistic 5.0 software (Esumi Co., Ltd.; Tokyo, Japan). One-way ANOVA was used to assess differences among groups, and individual means were compared by Tukey's multiple-range test. Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Effect of drying on survival of bacterial cells on the surface

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 sigma factors produced in the stationary phase lead to the generation of self-defense proteins that protect the cells against acidic conditions, desiccation, low temperature, and reactive oxidants (Spector and Kenyon, 2012). Therefore, we used stationary-phase bacterial cells in previous studies (Kuda et al., 2011, 2012; 2013) as well as in this study.

The viable count of the bacterial cells before and after drying on the stainless steel surface is summarized in Fig. 1. During the 120-min ventilation period at room temperature, viable cells of *Salmonella* Typhimurium in DW were dried thoroughly and the cell count decreased from approximately 8.1 log cfu/dish to 3.4 log cfu/dish on the stainless steel surface (Fig. 1A). On the other hand, *S. aureus* and *L. monocytogenes* showed resistance to desiccation in contrast to *Salmonella* Typhimurium (Fig. 1B and C). The food samples of 50% carrot juice and 5% *nori* solution, milk, and soy milk clearly protected the cells from desiccation.

The images of the dried and adhered cells on the cover slip observed by the AFM in dynamic force mode are shown in Fig. 2A–C. In this study, the gram-negative *Salmonella* Typhimurium cells were flattened by desiccation (Fig. 2A); however, the gram-positive *S. aureus* and *L. monocytogenes* cells retained their cell shape (Fig. 2A and B). This observation correlates with our and other previous reports regarding survival rates of gram-positive and -negative pathogens (Kuda et al., 2011; Pérez-Rodríguez et al., 2013).

3.2. Protective effect of decimal diluted food residues on the adhered pathogens

In Fig. 3, the horizontal lines marked as "DW" shows the survival cell counts of pathogens adhered with DW. Although the 100-fold diluted *nori* solution did not affect the survival rate of *Salmonella* Typhimurium, the 1000-fold diluted carrot juice, milk, and soy milk samples provided protection to *Salmonella* Typhimurium (Fig. 3A). *S. aureus* and *L. monocytogenes* were protected by the 100-fold diluted carrot juice and *nori* samples (Fig. 3B and C) as well as the 1000-fold diluted milk and soy milk samples.

Of particular note is the finding that only 100 nL (10^{-3} of 0.1 mL) milk and soy milk could protect 10% of the gram-positive bacterial cells from desiccation. In the case of *S. aureus*, the protection capacity of the 100-fold diluted *nori* sample was higher than that of carrot juice. This finding was not in agreement with the results from *Salmonella* Typhimurium. In the case of *L. monocytogenes*, the protective effect from carrot juice and *nori* samples was similar.

In the AFM observation, the adhered and dried pathogenic bacterial cells were not detected with the 50% carrot and 5% *nori* solutions, undiluted milk, and undiluted soy milk (Fig. 2D–G). Furthermore, 10% milk and 10% soy milk also covered the pathogenic cells (image not shown). Although the cells adhered with 10-fold diluted carrot and *nori* samples as well as 100-fold diluted milk and soy milk samples were detected, the cells were covered with food residue (Fig. 2H–K). Interestingly, *L. monocytogenes* showed an aggregation of food residue particles around the cells (Fig. 2H,K, and L). *Salmonella* Typhimurium cells adhered with 1000-fold diluted milk and soy milk samples were not flattened (Fig. 2M–O). AFM is used to observe the effect of disinfectants such as quaternary ammonium compounds on pathogens (Crismaru et al., 2011), as this is considered a powerful tool for nano-level analysis of the utensil surface-adhered microorganisms. In addition, observations could be made in real time, without pretreatments such as fixation, dehydration, or metal-coating, as are required for scanning electron microscopy. In this study, cover slips were used for AFM observation instead of stainless steel. In future, we would like to kinetic study with the actual stainless surface.

Milk and soy milk are rich in protein (approximately 33 mg/g and 38 mg/g, respectively), lipids (38 mg/g and 20 mg/g,

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