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Resistances to benzalkonium chloride of bacteria dried with food elements on stainless steel surface

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Abstract

To confirm the importance of washing food sediments from the surface of food-related environments, we examined resistances against benzalkonium chloride of pathogenic bacterial (*Escherichia coli* O26, *Pseudomonas aeruginosa* and *Staphylococcus aureus*) cells dried and adhered on stainless steel dishes with milk, beef gravy or tuna gravy. Suspensions (0.1 ml) of these bacteria (8–9 log cfu/ml) were put on a 5 cm φ stainless steel dish and dried at room temperature (20–24 °C) for 90 min in a bio-clean bench with ventilation. Though these bacteria suspended with distilled water decreased 30–40 fold during the drying period, milk and the gravies protected the bacteria. Without the food elements, the adhered *E. coli* and *Stap. aureus* were decreased from 6 to <2 log cfu/dish by 0.5 mg/ml benzalkonium chloride (BKC) for 10 min treatment. Although *Ps. aeruginosa* showed resistance to BKC, the adhered cells were inactivated by 2.0 mg/ml BKC. However, the bactericidal effect disappeared by the food elements, particularly with milk, even at 1.0 and/or 2.0 mg/ml BKC levels. The protective efficiency of milk on bacteria disappeared if washed with water.
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Keywords: Food sediment; Stainless steel; Benzalkonium chloride; *Escherichia coli*; *Pseudomonas aeruginosa*; *Staphylococcus aureus*

1. Introduction

Adhesion of microorganisms to equipment surface have the potential to transmit pathogens to food, and this is apparent in the food processing industry (Barnes, Lo, Adams, & Chamberlain, 1999; Giaouris & Nychas, 2006) and in the domestic environment (Humphrey, Martin, Slader, & Durham, 2001). There are many kinds of disinfectants for food utensils, such as alcoholic solutions and hypochloric solutions. Quaternary ammonium compounds are cationic biocides that are commonly used as disinfectants in food production environments (Krysinski, Brown, & Marchisello, 1992). Benzalkonium chloride (BKC) is a quaternary ammonium compound that is widely used for sanitation in food-processing environments (Mustapha & Liewen, 1989). BKC acts on general membrane permeability, causing the cytolytic leakage of cytoplasmic materials at low concentrations. At high concentrations, they target the carboxylic groups and cause general coagulation in the bacterial cytoplasm (To, Favrin, Romanova, Mansel, & Griffiths, 2002). Previous reports showed that these bacteria have different resistivities (*Pseudomonas aeruginosa* > *Escherichia coli* > *Staphylococcus aureus*) against BKC in suspensions (Reuda, Amigot Lázaro, & Ducha, 2003).

It is known that the microorganisms on the inner surfaces of food and medical apparatuses and equipments often forms biofilm, and it is reported that the tolerance of the biofilms to various stresses is different from the planktonic cells in the test tube (Carpentier & Cerf, 1993). Particularly, there are many reports about the resistances of biofilms of *Ps. aeruginosa* (Ishikawa & Horii, 2005; Landry, An, Hupp, Singh, & Parsek, 2006), *Stap. aureus* (Shanks et al., 2005; Valle et al., 2003) and *Listeria monocytogenes* (Carpentier & Chassaing, 2004; Chemielewski & Frank, 2006) because of their strong resistances against disinfectants and for serious medical reasons, such as nosocomial infections.

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On the other hand, it is considered that the food elements protect microbial cells when the adhered cells are dried on the surface of food utensils and equipments (Leslie, Israeli, Lighthart, Crowe, & Crowe, 1995), and maybe the microorganisms achieve resistances to disinfectants and biofilms. Although there are many reports about the effect of dirty conditions determined by the European Standard EN-1276: 1997 (Payne, Babb, & Bradley, 1999; Taylor, Rogers, & Holah, 1999), these studies were carried out in suspensions in tubes.

In this study, to determine the importance of washing food sediments from the surface of food utensils before chemical disinfection, the protective efficiency of milk, beef gravy and tuna gravy on three pathogenic bacteria (*E. coli* O26, *Ps. aeruginosa*, *Stap. aureus*), they were dried and adhered on stainless steel dishes were examined.

2. Material and methods

2.1. Bacterial culturing

E. coli O26:HNM (VT1), *Ps. aeruginosa* IAM1514 and *Stap. aureus* IAM 12544 were employed to investigate attachment and disinfection treatments on stainless steel surfaces. To produce cultures, the bacterial cells were inoculated into 5 ml of Trypton-Soya Broth (TSB, Nissui Pharmaceutical Co., Tokyo, Japan) and incubated at 37 °C for 18 h with shaking (120 rpm). The culture reached the stationary phase.

2.2. Chemicals and food materials

Ten percent BKC was purchased from Wako Pure Chemical (Osaka, Japan). Alkyl ether sulfuric acid ester sodium (AES), a neutralized detergent, was purchased from Kao Corporation (Tokyo). Ultra high temperature-treated (UHT) milk, frozen beef (outside round) meat and yellowfin tuna *Thunnus albacares* meat were purchased from retail shop. Beef gravy and tuna gravy were prepared from same volumes of the meat and distilled water (50% gravies). These were minced and centrifuged (2000*g* for 10 min at room temperature).

2.3. Preparing of stainless steel dishes

Commercial stainless steel dishes (sus304, 5 cmφ, As-one Co., Tokyo) were used in this study. In advance, the dishes were carried out the twice treatment of ultrasonication for 15 min, brushing for 60 s and autoclaving at 121 °C for 15 min.

2.4. Bacterial adhesions

Bacterial suspensions were prepared from stationary phase cultures (5 ml). These were centrifuged at approximately 2000*g* for 5 min at room temperature (Decleva, Menegazzi, Busetto, Patriarca, & Dri, 2006) and resuspended in 0.31 mmol/l phosphate-buffered saline (PBS, pH 7.2). This washing process was carried out twice. The cells were finally resuspended in 5 ml of distilled water, milk, 50% beef gravy or 50% tuna gravy. The cell concentration was 9.0–9.3 log cfu/ml. Bacterial suspension (0.1 ml) was placed on the center of the stainless steel dish ($n = 3$) and dried for 90 min at room temperature (20–24 °C) in a bio-clean bench (SCB-1300B, Shimadzu Rika Instrument, Tokyo) with ventilation (20 m^3/min). After drying, the cells adhered as a dried scale with about a 10 mm-diameter circle.

2.5. BKC treatment and enumeration of survival cells

To determine the bactericidal effect of BKC, the dried and attached cells were covered with 0.1 ml of 0, 0.5, 1.0 and 2.0 mg/ml BKC solution. After 10 min at room temperature, 5 ml of TSB was added. Then, the attached cells were brushed and suspended well for 60 s using cotton swab (for microbial test, Nissui Pharmaceutical Co., Tokyo) and this suspension (0.1 ml) was plated immediately on a Tripton Soya Agar (TSA, Oxoid, Basingstoke, UK). The incubation was carried out at 37 °C for 24 h.

Data of the survival cells on stainless steel dish were expressed as the mean and SD or SE of log cfu/dish ($n = 3$). Statistical analysis was performed using the software EXCEL Statistic 5.0 (Esumi Co., Ltd., Tokyo). One-way ANOVA was used to assess the differences. Then, individual means were compared by Duncan's multiple-range test or Student's *t*-test. Significant differences were accepted at $p < 0.05$.

2.6. Effect of water washing on adhered cells with milk before the BKC treatment

To determine the effect of water washing, after the cell adhesion with milk on a stainless steel surface in the same way as above, water washing treatment was carried out as

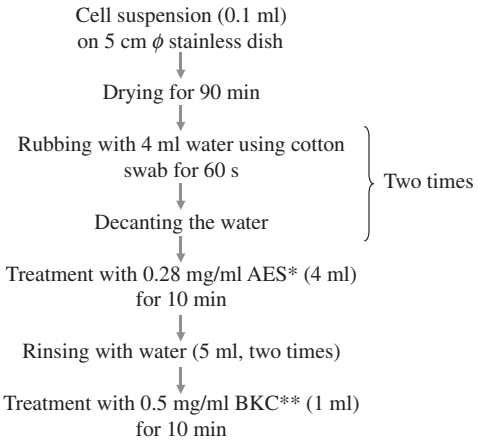

Fig. 1. Scheme of the method for the effect of water washing on adhered cells with milk before benzalkonium chloride treatment. *Alkyl ether sulfuric acid ester sodium, **benzalkonium chloride.

shown in Fig. 1. This method was modeled on domestic and food service environments. The adhered cells were washed twice using cotton swab for 60 s with 4 ml of water. Then survival cells on a stainless steel surface were treated with 4 ml of 0.28 mg/ml AES for 10 min. After water rinsing (5 ml, twice), 0.5 mg/ml BKC (1 ml) was added and incubated for 10 min. Finally, the BKC-treated dishes were rinsed twice again with 5 ml of water. After each treatment, survival cells were counted in the same way as above.

3. Results and discussion

3.1. Effect of drying on bactericidal cells

Fig. 2. shows the effect of drying at room temperature with ventilation for 90 min on *E. coli* O26, *Ps. aeruginosa* and *Stap. aureus* cells prepared from different growth stages. Sensitivities of the logarithm and early stationary growth phase cells to the dryness were higher than the ones of the stationary growth phase cells. It has been reported that sigma factors differed by logarithm and stationary growth phases and they produce different proteins (Siegele & Kolter, 1992). The sigma factors produced in the stationary phase lead proteins that protect own cells from acid, dryness, low temperature and peroxidants (Pohlmann-Dietze et al., 2000; Valle et al., 2003). Perhaps, most bacteria in the general environment exist as stationary phase cells rather than as logarithm growth phase cells, because the general environments were not optimal for bacterial growth. Therefore, we used stationary phase bacterial cells in this study.

3.2. Efficiency of food elements on bacterial cells adhered on stainless steel surface

Table 1 summarizes the disinfection effect of BKC and the protection efficiency of food elements on *E. coli* cells dried on stainless steel surface. Even with 0.5 mg/ml BKC, it showed bactericidal effect without food elements. However, the food elements, particularly milk, prevent the bactericidal effect. Even with 2.0 mg/ml BKC level, which is the upper level for sanitation of food utensils and environment, the viable count of cells dried with milk was decreased only from 7.54 to 7.15 log cfu/dish.

*Ps. aeruginos*a cells showed the highest resistance against BKC among the three bacteria used in this study (Table 2). There are many reports about the resistance of *Ps. aeruginosa* against BKC and other disinfectants (Loughlin Jones, & Lambert, 2002; Sakagami Yokoyama, Nishimura, Ose, & Tashima, 1989). Nevertheless, 2.0 mg/ml of BKC could reduce both the planktonic and adhered cells without food elements. Not only milk but also meat gravies prevent the bactericidal activity of BKC.

Although the sensitiveness of *Stap. aureus* to BKC was higher than the ones of *E. coli* and *Ps. aeruginosa* (Reuda et al., 2003), the food elements also decreased the BKC efficiency (Table 3). It is thought that gram positive bacteria rather than gram negative bacteria were sensitive to BKC (Brill, Goroncy-Bermes, & Sand, 2006). However, biofilm of *Stap. aureus* have resistance to various stresses including disinfectants (Amorena et al., 1999; Luppens, Reij, van der Heijden, Rombouts, & Abee, 2002).

According to the Standard Tables of Food Composition in Japan (2007), the cell suspensions with food elements may contain protein, fat and carbohydrate as below. Milk contains about 3 g of proteins, 4 g of fats and 5 g of carbohydrates per 100 ml. Beef gravy may contain about 10 g of proteins, 2 g of fats per 100 ml and less carbohydrate. Tuna gravy may contain about 12 g of protein and only less fat and carbohydrate. Leslie et al. (1995) reported that the disaccharides trehalose and sucrose protect both membranes and proteins in intact *E. coli* during drying. Banin, Brady, & Peter Greenberg (2006) indicated that magnesium, calcium and iron protect *Ps. aeruginosa* biofilms against EDTA treatment. Truby and Bennett (1966) suggested that the role of lipid in the protection of *Stap. aureus* against trichlorophenol is because of the hydrogen bonding between lipid and cells, and/or between

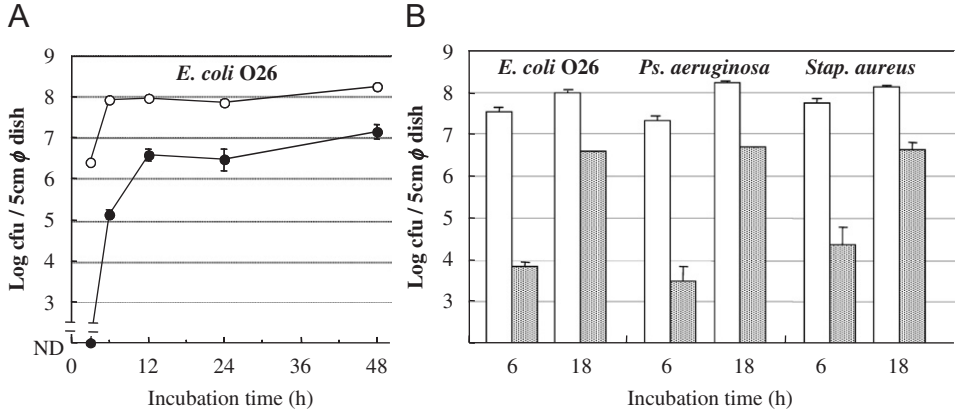

Fig. 2. Resistance of different growth phase bacterial cells against dryness. (A) *E. coli* O26 cells prepared from 3 to 48 h incubation periods (open circles) and were dried on stainless steel with ventilation for 90 min (closed circles). (B) Three bacterial cells were prepared from 6 and 18 h incubation periods (open column) and were dried in the same way as above (closed column). Values are mean and SE of log cfu/dish ($n = 3$).

Table 1
Effects of food elements on the bactericidal efficiency of BKC on *E. coli* O26 adhered on a stainless steel surface (log cfu/dish)

BKC concentration (mg/ml)	Before drying	Dried with			
		DW	Milk	Beef gravy	Tuna gravy
0	7.99±0.06	6.35±0.45 c	7.54±0.05 b	7.85±0.03 a	7.85±0.06 a
0.5	ND	ND	7.73±0.04 a	7.41±0.14 b	7.62±0.20 b
1.0	ND	ND	7.19±0.09 a	6.57±0.16 b	6.04±0.16 c
2.0	ND	ND	7.15±0.23 a	4.49±0.20 b	ND

Cell suspensions (0.1 ml) were placed onto a stainless steel surface and dried in a clean bench for 90 min with ventilation. Values are mean and SD ($n = 3$).
DW, distilled water; ND, not detected (<2.0).
Data of dried samples in the same line with different letters are significantly different ($p < 0.05$) among treatments.

Table 2
Effects of food elements on the bactericidal efficiency of BKC on *Ps. aeruginosa* adhered on a stainless steel surface (log cfu/dish)

BKC concentration (mg/ml)	Before drying	Dried with			
		DW	Milk	Beef gravy	Tuna gravy
0	8.25±0.06	6.69±0.03 c	7.43±0.31 b	7.88±0.03 a	7.85±0.07 a
0.5	6.46±0.33	5.12±0.28 c	7.19±0.08 b	7.38±0.18 ab	7.34±0.12 a
1.0	5.98±0.39	2.94±0.82 b	7.23±0.22 a	7.27±0.03 a	6.87±0.15 a
2.0	2.65±0.16	ND	7.01±0.05 b	7.14±0.05 a	6.62±0.09 c

See footnotes of Table 1.

Table 3
Effects of food elements on the bactericidal efficiency of BKC on *Stap. aureus* adhered on a stainless steel surface (log cfu/dish)

BKC concentration (mg/ml)	Before drying	Dried with			
		DW	Milk	Beef gravy	Tuna gravy
0	8.13±0.08	6.63±0.03 d	8.12±0.05 a	7.88±0.02 c	8.08±0.04 b
0.5	ND	ND	8.01±0.10 a	7.34±0.04 b	7.02±0.16 c
1.0	ND	ND	7.18±0.03 a	5.97±0.39 b	7.06±0.10 a
2.0	ND	ND	5.62±0.86 a	4.11±0.67 ab	4.88±0.60 b

See footnotes of Table 1.

lipid and the phenol compound. In this study, the protection by each food was different depending on the kind of bacteria. Although it is thought that this difference is caused by the difference of the main elements of the food materials and the structures of cell membrane and s-layers, more detailed examinations are necessary.

3.3. Effect of water washing on adhered cells with milk before BKC treatment

Water washing twice with cotton swab detached the bacterial cells from the stainless steel surface and the survival cell count on the dish was decreased about 80–300 fold without milk and 30–40 fold with milk (Table 4). After detergent treatment for 10 min and washing, the bacterial, particularly *Ps. aeruginosa*, cells were detached from the stainless steel surface. Then the survival cell counts on the dishes could not be detected by 0.5 mg/ml BKC treatment for a 10 min treatment. This BKC concentration could not affect the bacteria dried with milk without washing (Tables 1–3).

As with previous reports (Adair, Geftic, & Gelzer, 1969; Brill et al., 2006; Shih & Huang, 2002), *Ps. aeruginosa* showed resistance to BKC. Furthermore, the food elements prevented the BKC efficiency on *Stap. aureus,* although gram positives were regarded as BKC sensitive. The situations that microbes dried with food elements are considered to occur in the food industry and in the domestic kitchen environment. It may introduce problems, such as cross contamination and producing a base for biofilms. There are many reports about biofilms including detaching methods and disinfection methods (Krysinski et al., 1992; Stewart, 2002). They show that once biofilms were created, the detaching and sterilization are difficult.

Table 4
Effect of common washing on adhered pathogenic bacteria with milk

Washing process	*Escherichia coli* O26 dried with		*Pseudomonas aeruginosa* dried with		*Staphylococcus aureus* dried with	
	Distilled water	Milk	Distilled water	Milk	Distilled water	Milk
A: Before drying	7.99±0.06		8.25±0.06		8.13±0.08	
B: After drying	6.58±0.05	7.70±0.11	5.64±0.23	7.38±0.07	6.32±0.32	7.73±0.04
C: After water washing	4.70±0.05	6.20±0.35	3.17±0.30	5.79±0.14	4.03±0.20	6.23±0.09
C + water washing						
D: without detergent	3.16±0.32	4.54±0.09	ND	3.82±0.27	2.28±0.27	4.80±0.27
E: with detergent	2.70±0.87	4.22±0.28	ND	3.89±0.17	ND	4.67±0.22
BKC treatment						
F: D + 0.5 mg/ml BKC (1 ml) for 10 min	ND	ND	ND	ND	ND	ND
G: E + 0.5 mg/ml BKC (1 ml) for 10 min	ND	ND	ND	ND	ND	ND
Water rinsing						
H: F + rinsing	ND	ND	ND	ND	ND	ND
I: G + rinsing	ND	ND	ND	ND	ND	ND

See footnotes of Table 1.
B: Cell suspensions (0.1 ml) were placed onto 5 cmφ stainless dish and dried in a clean bench for 90 min.
C: Twice treatment of brushing using cotton swab for 60 s with 4 ml of distilled water.
E: 0.285 mg/ml alkyl ether sulfuric acid ester sodium (AES) treatment for 10 min.
H and I: Twice rinsing with 4 ml of distilled water.

Therefore, we think the results of this study confirm the importance of daily adequately washing and the disinfection treatment for prevent cross contamination and biofilm formation in food-related environments.

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