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The Disinfectant Effects of Benzalkonium Chloride on Some Important Foodborne Pathogens

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Abstract: Antimicrobial chemicals are widely applied in the food processing industry. In attention to extent uses of disinfectants, it is necessary to evaluate and certificate the efficacy of disinfectants and employing the minimum effective dosages. In this research the antibacterial influences of a common disinfectant solution used in food industry, benzalkonium chloride that is a quaternary ammonium compound, were evaluated on six important foodborne pathogens including three Gram positive (*Staphylococcus aureus, Listeria monocytogenes* and *Bacillus cereus*) and three Gram negative bacteria (*Salmonella typhimurium, Escherichia coli* and *Pseudomonas aeruginosa*). According to the obtained results after three times conducing experiments, *Listeria monocytogenes* and *Bacillus cereus* were the most sensitive and resistant studied bacteria with MIC and MBC equal to 30 & 35 mg/L and 140 & 160 mg/L respectively. In attention to obtained results, the used disinfectant has good antibacterial effects. Benzalkonium chloride was more effective on Gram positive than Gram negative bacteria, except *Bacillus cereus* because of its ability to spore formation. In attention to extent resistance towards quaternary ammonium compounds, further studies have to be done to verify if the bacteria develop resistance toward common disinfectants such as the benzalkonium chloride.

Key words: Disinfectant · Benzalkonium Chloride · Bacteria · Foodborne Pathogens

INTRODUCTION

Food provides an excellent environment for microorganisms to grow [1]. Adhesions of microorganisms to equipment surface have the potential to transmit pathogens to food and this is apparent in the food processing industry [2-4] and in the domestic environment [5]. Therefore surfaces of equipment used for food handling and processing are recognized as sources of microbial contamination and recontamination. Recontamination is the primary source of bacterial pathogens in many commercially prepared ready to eat food [6]. The number of foodborne illness outbreaks linked to the presence of pathogenic microorganisms on fresh produce has increased over the past years. Possible contamination sources are soil, feces, manure, irrigation and washing water, animals (including insects and birds), product handling, harvesting and processing equipment and transport [7-9].

In the food industry, regular cleaning and disinfection procedures are the most effective way of controlling levels of pathogenic microorganisms that are prevalent in food and the environment and have the potential to cause serious illnesses [10, 11]. The cleaning procedures involve the use of detergents, which are not designed as antimicrobial agents but to break down food soils and remove surface contamination, followed by applications of disinfectants that reduce the viability of the remaining organisms [11].

There are many kinds of disinfectants for food utensils, such as alcoholic solutions and hypochloric solutions including sodium hypochlorite, peracetic acid, alcohol based products and quaternary ammonium compounds (QACs) such as benzalkonium chloride (BAC), a synthetic antimicrobial agent with a broad spectrum antimicrobial [11-13]. The QACs are amphoteric surfactants generally contain one quaternary nitrogen associated with at least one major hydrophobic

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substitute [14]. QACs are cationic biocides that are commonly used as disinfectants in food production environments [15]. BAC is a QAC that is widely used as disinfectant and cationic surface active agent for sanitation in food processing lines and surfaces in the food industry [4], as clinical disinfectant and antiseptic (topical) in health care facilities and domestic households and as antimicrobial preservative in drugs in low concentration [16, 17]. BAC is the product of a nucleophilic substitution reaction of alkyldimethylamine with benzyl chloride [17].

QACs are active against bacteria and some viruses, fungi, yeasts and protozoa. The solutions are bacteriostatic or bactericidal according to their concentration [1]. They may provide effective treatment in bacterial removal without affecting the structural integrity of produce [18]. BAC acts on general membrane permeability, causing the cytolytic leakage of cytoplasmic materials at low concentrations with damage to the outer membrane and promote their own intracellular uptake and entry. At high concentrations, they target the carboxylic groups and cause general coagulation in the bacterial cytoplasm [19, 20]. Their surfactant ability and positive charge provide a great capacity to penetrate and adhere to porous surfaces [14, 21]. The outermost surface of bacterial cells universally carries a net negative charge, often stabilized by the presence of divalent cations. This is associated with the teichoic acid and polysaccharide elements of Gram positive bacteria, the lipopolysaccharide of Gram negative bacteria and the cytoplasmic membrane itself. It is not therefore surprising that many antimicrobial agents are cationic and have a high binding affinity for bacterial cells. Often, cationic antimicrobials require only a strong positive charge together with a hydrophobic region in order to interact with the cell surface and integrate into the cytoplasmic membrane [14].

To assess the suitability of an antiseptic agent, both the microbicidal activity and the cytotoxic effect must be taken into consideration to select biocompatible antibacterial agents [22]. BAC based disinfectants are nontoxic, non tainting and odor free at use dilutions and compatible with nonionic, ampholytic and cationic surface active agents. BAC is widely used in the formulation of cleaner and sanitizers for dairy industry, food storage tanks, catering industry and fisheries. Health care associated infections most commonly result from person to person transmission via the hands of workers. Topical applications of QACs remain safe and effective preventative and treatment measures [14]. BAC hand sanitizer is the most popular rinse free hand sanitizer formula for normal hand washing [23]. Also intranasal products containing the preservative BAC appear to be safe and well tolerated for both long term and short term clinical use [24].

Cationic antimicrobials including the QACs have been in general use within clinical and domestic settings for over half a century. Recently, the use of antiseptics and disinfectants has been questioned in such settings because of the possibility that chronic exposure of the environment to such agents might select for less susceptible strains towards these agents [14, 25, 26]. Resistance to disinfectants based on QAC is a potential problem in the food processing industry [27]. Concerns about bacterial resistance have led to calls for increased education of both public and professionals on the correct use of disinfectants and antibiotics. Additionally, more stringent infection control measures have been advocated in order to reduce the transmission of infection [14, 28, 29].

In the present study a conventional antiseptic agent, BAC was applied against some food pathogenic microorganisms and the bacterial effectiveness of it was evaluated against some important foodborne Gram positive (*Staphylococcus aureus, Listeria monocytogenes* and *Bacillus cereus*) as well as Gram negative (*Salmonella typhimurium, Escherichia coli* and *Pseudomonas aeruginosa*) pathogens.

MATERIALS AND METHODS

Materials, Bacterial Strains and Culture Conditions: Ten percent commercial BAC was purchased from the market. *Staphylococcus aureus* (ATCC 6538), *Listeria monocytogenes* (ATCC 19118) and *Bacillus cereus* (ATCC 11778) as Gram positive and *Salmonella typhimurium* (ATCC 14028), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 25619) as Gram negative bacteria were used. For the antimicrobial activity measurement, first of all active cultures were generated by inoculating a single colony of each bacterium into 5 ml sterile nutrient broth (Merck) and incubating at 37°C for 24 h. Freshly synchronized cultures of bacterial strains forming initial inoculums were prepared after two times overnight cultures (24 h) of each bacterium by successively transferring 100 µl of the vegetative cells into tryptic soy broth (TSB, Merck). Then the optical densities of the active freshly synchronized cultures were adjusted at 600 nm to a cell density equivalent to 10^6 CFU/mL [30, 31].

Table 1: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of BAC for the Gram positive bacteria

Gram- positive bacteria	*S. aureus*	*L. monocytogenes*	*B. cereus*
MIC	40 mg/L	30 mg/L	140 mg/L
MBC	45 mg/L	35 mg/L	160 mg/L

Table 2: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of BAC for the Gram negative bacteria

Gram-negative bacteria	*S. typhimurium*	*E. coli*	*P. aeruginosa*
MIC	80 mg/L	40 mg/L	60 mg/L
MBC	100 mg/L	45 mg/L	80 mg/L

Measurement of Antibacterial Activity of BAC: In order to evaluate the antimicrobial activity by the tube dilution method, BAC stock solution was serially diluted with TSB to obtain test solutions containing required concentrations (from 5 until 600 mg/L) of BAC. Then aliquots of 1ml from each bacterium (about 10^6 CFU/mL) were added to the tubes containing 1mL of the prepared concentrations of BAC solutions separately. At the end of contact time (24 h incubation at 37°C), the tubes were examined for growth and then the amount of MIC and MBC were determined. The MIC is defined as the lowest concentration of BAC showing no visible bacterial growth after the incubation time [32- 35]. Then in order to measure bacterial viability, 100 µL from tubes that showed no visible growth were spread on the standard plate count agar plates and incubated for 24 h at 37°C. The MBC is defined as the lowest concentration of BAC at which no visible bacterial growth (colony) is observed after subculturing [32]. All the above mentioned assays were run three times in parallel with negative and positive controls.

RESULTS

According to the obtained results as shown in Table 1, *Listeria monocytogenes* showed the maximum sensitivity to BAC among the studied bacterial species with MIC and MBC equal to 30 and 35 mg/L respectively. *Staphylococcus aureus* and *Escherichia coli* were proved to be the second sensitive microorganisms with MIC and MBC equal to 40 and 45 mg/L respectively. Table 1 shows that *Bacillus cereus* was the most resistant bacteria in this study with MIC and MBC equal to 140 and 160 mg/L, respectively. The results of present study showed that BAC in 160 mg/L concentration was able enough to kill all target bacteria during 24 h contact time.

DISCUSSION

In present study, BAC showed strong antibacterial activity and according to the results *Listeria monocytogenes* and *Bacillus cereus* were the most sensitive and resistant studied bacteria respectively. The studied Gram positive bacteria were more sensitive than Gram negative ones, except *Bacillus cereus* because of its ability to spore formation. Similar to our research, Heinzel [36] reported that spore forming bacteria such as *Bacillus cereus* are considered to be resistant to BAC. Many studies have investigated BAC and its influence on various microorganisms in different conditions. In many studies Gram positive bacteria were more susceptible than Gram negative [1, 24]. In Gram negative bacteria, resistance mechanisms are more complicated since these organisms possess an inner and an outer membrane. The latter membrane has a clear role in modulating the accessibility of a cell to preservatives and other small molecules; the lipopolysaccharide layer is of crucial importance in this respect [37, 38]. Previous reports showed that these bacteria have different resistance (*Pseudomonas aeruginosa* > *Escherichia coli* > *Staphylococcus aureus*) against BAC in suspensions [36]. Although biocides can generally be regarded to act non-specific and or multifactorial at use concentrations [40], resistance to biocides is of interest for the medical area, especially if a cross resistance to antibiotics is observed, as reported for *Staphylococcus* spp. isolated from the food industry and also for *Pseudomonas aeruginosa* for QACs [41].

The sanitizing activity of benzalkonium chloride on planktonic cells and biofilms has been previously studied [18, 42]. Numerous authors have demonstrated that biofilm formation or bacterial attachment [18, 43] increases resistance to several physicochemical stresses such as antibacterial agents and pH [44]. Thus, biofilms may be a niche in which pathogens survive the regular cleaning and sanitation procedures and persist in food processing environments [42]. Biofilm formation was positively correlated with resistance to QACs [14, 45]. Growth as a biofilm also reduces the susceptibility profile and is probably caused by a variety of factors including nutrient depletion within the biofilm, reduced access of the biocide to cells in the biofilm, chemical interaction between the biocide and the biofilm and the production of degradative enzymes and neutralizing chemicals [46, 47]. Biofilm formation is thought to play an important role in the survival of virulent strains of food related

staphylococci. Staphylococci isolated from the food industry are found to vary greatly in their ability to form biofilms [45]. It is thought that Gram positive bacteria rather than Gram negative bacteria were sensitive to BAC [48]. However, biofilm of *Staphylococcus aureus* have resistance to various stresses including disinfectants [49]. It is known that the microorganisms on the inner surfaces of food and medical apparatuses and equipments often form biofilm [4]. Particularly, there are many reports about the resistances of biofilms of *Pseudomonas aeruginosa* [50] and *Staphylococcus aureus* [44, 51] because of their serious medical reasons. On the other hand in our results, *Staphylococcus aureus* and *Escherichia coli* showed a similar sensitivity to BAC that may be due to ability of *Staphylococcus aureus* to formation biofilm and cross resistance.

It was shown earlier that *Listeria monocytogenes* is sensitive to BAC with MIC equal to 0.78 µg/ml [52]. Another study was conducted to determine the susceptibility of 114 *Listeria monocytogenes* isolated from food products to BAC by MICs. The MICs for benzalkonium chloride formed a bimodal distribution, with 105 isolates having a MIC of 4 mg/L and 9 isolates with MICs of 16 and 32 mg/L [53] which is nearly the same as our obtained result equal to 30 mg/L. Some studies similar to this study showed that some isolates of *Listeria monocytogenes* can form biofilm that are resistant against disinfectants [42].

A total of 569 different bacterial isolates (*Salmonella*, *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus hyicus*, *Enterococcus faecalis*, *Enterococcus faecium*) were tested for susceptibility to BAC using MIC determination. Staphylococci were in general very susceptible to benzalkonium chloride. The *Salmonella* isolates were in general less susceptible followed by *Escherichia coli* and the Gram positive species [54]. The antibacterial effect of six pure QAC disinfectants on clinical isolates of *Salmonella typhimurium, Serratia marcescens* and *Pseudomonas aeruginosa* was studied. The antibacterial efficacy of disinfectants on *Salmonella typhimurium* in the study is the highest in comparison with *Serratia marcescens* and *Pseudomonas aeruginosa* strains [55]. Also some species of bacteria, notably *Pseudomonas aeruginosa*, are relatively insensitive to QAC biocides. This is thought to relate to a failure of the compounds to penetrate the outer membrane and to access the cytoplasmic membrane [14]. Kuda *et al*. [4] examined the resistance of pathogenic bacterial (*Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*) cells dried and adhered on stainless steel surface against BAC. The results showed the adhered *Escherichia coli* and *Staphylococcus aureus* were decreased from 2 to 6 log CFU/dish by 0.5 mg/ml BAC for 10 minutes treatment. Although *Pseudomonas aeruginosa* showed resistance to BAC, the adhered cells were inactivated by 2.0 mg/ml BAC. In a research BAC in 50 µg/ml would only be effective in eliminating *Staphylococcus aureus* and coagulase-negative staphylococci for one hour but not *Pseudomonas aeruginosa* till 8 hours [56]. These results are in accordance to the sensitivity of studied bacteria in our obtained results.

In conclusion, the risk of hazards in food production and processing caused by resistance to biocides is regarded as low, as long as biocides are used under appropriate conditions. Rotational use of different disinfectants is recommended to avoid development of resistance or selection of resistant strains in an environment which is frequently disinfected.

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