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Chlorhexidine Gluconate Tolerance by *Acinetobacter* spp. Isolated from Foods Originated from Brazil

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HIGHLIGHTS

- Isolates from goat's milk were sensitive to low concentrations of Chlorhexidine Gluconate (CG).
- Minimum Inhibitory Concentration of CG for some *Acinetobacter* isolates from salads was similar to that of clinical isolates.
- Despite being effective, CG should be used sparingly for food handlers.

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Acronyms and abbreviations
CG=Chlorhexidine Gluconate
MBC=Minimum Bactericidal Concentration
MIC=Minimum Inhibitory Concentration

ABSTRACT

Background: In recent years, *Acinetobacter* spp. have emerged as opportunistic food-borne pathogens worldwide. The purpose of this study was to evaluate the tolerance to chlorhexidine by *Acinetobacter* spp. isolated from foods that are handled and consumed without any prior heat treatment.

Methods: Eleven *Acinetobacter* spp. isolates from ready-to-eat salads and four from raw goat milk were previously collected. The samples were evaluated for tolerance to Chlorhexidine Gluconate (CG) based on the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC). The evaluation was performed using the dilution method in titration microplates. Statistical analysis by GraphPad software was performed using the t-test to compare the values.

Results: The MIC and MBC of CG varied according to the origin of the isolates. Goat milk *Acinetobacter* spp. isolates were inhibited at MIC and MBC of ≤7.8 ppm CG. For most *Acinetobacter* spp. isolated from salads, however, MIC and MBC values ranged between 31.2-62.5 ppm, which are values generally correlated with clinical isolates. An MIC of 250 ppm was verified for only one isolate (F2R21).

Conclusion: Even food isolates can present MIC and MBC values for CG comparable to those of multidrug resistant isolates from clinical origin, suggesting that this sanitizer should be used sparingly for food handlers.

Introduction

Some species from the genus *Acinetobacter*, such as *A. baumannii* complex, are considered important pathogens in hospital settings and other healthcare units. However, in recent years, several studies have described the isolation of antibiotic-resistant strains of *Acinetobacter* spp. in food. These findings suggest that food of both animal and plant origin may be vectors of infectious bacteria, which is a cause for great concern as it would impact public health due to the potential for the transmissions occurring outside the health care units

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(Elbehiry et al., 2021; Malta et al., 2020).

Workers in the food sector have been implicated in various outbreaks of food-borne diseases due to cross-contamination of ready-to-eat foods with contaminated raw ingredients and hands acting as the main vectors of pathogen transfer (Margas and Holah, 2014; Todd et al., 2010). Therefore, proper hand hygiene is fundamental for avoiding the spread of pathogens.

Chlorhexidine Gluconate (CG) is one of the products approved by the Food and Drug Administration for use as an antiseptic by food handlers. Food Handlers Antiseptics are defined as "antiseptic products intended for use by professional food handlers in commercial or regulated environments where food is grown, harvested, produced, manufactured, processed, packaged, transported, stored, prepared, served, or consumed" (FDA, 2020). CG is active against bacteria, some enveloped viruses, and fungi which are used in hand rubs, body washes, and even mouthwashes (Kampf, 2016; Leshem et al., 2022). This compound exerts bacteriostatic or bactericidal activity depending on the concentration. Its mode of action involves the binding of the positively charged CG molecules to the negatively charged bacterial membranes including the cell wall (Horner et al., 2012). At low concentrations, their interaction with the bacterial cell membrane results in the loss of osmoregulatory and metabolic capabilities, leading to the loss of important ions from within the bacterial cell. At higher concentrations, CG results in the loss of membrane integrity and leakage of cellular contents, consequently leading to lysis and cell death (Horner et al., 2012; Leshem et al., 2022).

The tolerance of different hospital-derived bacteria to biocides, such as CG, has been reported previously (Kampf, 2016; Wand et al., 2017). However, few studies have described the tolerance of *Acinetobacter* spp. in food to sanitizing agents. Therefore, the objective of this study was to evaluate the tolerance to chlorhexidine exhibited by *Acinetobacter* spp. isolated from foods that are handled and consumed without any prior heat treatment such as milk and salads.

Materials and methods

Samples collection

In this study, 15 *Acinetobacter* spp. isolates were used. These isolates were obtained from raw goat milk (n=4) and ready-to-eat salads (n=11), and were identified using mass spectrometry (MALDI-TOF, Microflex LT, Bruker, United States) in previous studies. Ready-to-eat raw vegetable salads were acquired from different self-service restaurants in Niterói, Brazil. Sample collection was performed over a 13-month period from November 2017 to November 2018 (Beltrão, 2019). Raw goat milk was purchased directly from producers based in several regions of the state of Rio de Janeiro. These samples were collected over a period of five months between March to August 2018 (Ramos and Nascimento, 2019).

Samples preparation

Before commencing the experiments, the isolates were subcultured on Casoy agar (Merck, São Paulo, Brazil) from a frozen stock culture stored at -20 °C in Casoy broth (Merck, São Paulo, Brazil) containing 40% glycerol (Merck, Germany). The subcultures were incubated at 37 °C for 24 h.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC of CG was determined using a 2% solution (RIOHEX, Rioquímica, São Paulo, Brazil) and following the dilution method in titration microplates, as described by Obe et al. (2021). CG (200 µl) was added to the first well of the titration microplates, and 100 µl of Tryptic Soy Broth (TSB; Himedia, São Paulo, Brazil) were added to the remaining wells in the same row. CG was diluted by transferring 100 µl from the first to the last well. Colonies of each isolate were inoculated in 0.85% (w/v) saline solution until the turbidity was equivalent to McFarland's 0.5 scale (approximately 1.5×10^8 Colony Forming Unit (CFU)/ml). Then, 100 µl of this cell supension was added to each well, resulting in concentration of approximately 10^6 CFU per well, that was confirmed by plating. MIC was defined as the lowest concentration of sanitizer that inhibited the growth of each *Acinetobacter* spp. The final concentration of CG in the wells was 1.9-1,000 ppm. The experiment was repeated thrice for each isolate. An isolate was considered CG-tolerant, if it exceeded the cut-off value of 64 ppm, as defined by Morrissey et al. (2014).

Determination of Minimum Bactericidal Concentration (MBC)

The MBC was evaluated as described by Haubert et al. (2022). MBC was evaluated from wells without visible bacterial growth, as described in the previous experiment. Aliquots from these wells were cultured on TSA plates and incubated at 37 °C for 24 h. After incubation, the colonies were counted. Then, MBC was defined as the lowest concentration of sanitizer which resulted in the death of 99.9% of the initially inoculated cells.

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Statistical analysis

Differences in the MIC and MBC values between the isolates obtained from salads and raw milk were compared using unpaired t-test using GraphPad software. The *p* values <0.05 were considered significant.

Results and discussion

Fifteen isolates belonging to the *Acinetobacter* spp., namely five *A. baumannii*, five *A. nosocomialis*, and one *A. gerneri* were isolated from ready-to-eat salads. Two *A. guillauiae* and two *A. ursingii* isolates were obtained from raw goat milk. The tolerance to CG was evaluated in all the isolates. The MIC of CG was identical to that of MBC for all isolates, which ranged between 3.9-62.5 ppm (Table 1) for all 15 *Acinetobacter* spp. isolates. Interestingly, the four isolates from goat milk showed the lowest values (3.9-7.8 ppm). Statistical analysis, however, showed no significant difference between the MIC and MBC values presented by the two groups of isolates (p=0.14). Out of the 11 isolates from salads, the MIC and MBC values for CG for 10 isolates ranged from 31.2-62.5 ppm. The remaining isolate F2R21 identified as *A. baumannii* had corresponding MIC and MBC values of 250 ppm, which is a high value for CG.

Table 1: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Chlorhexidine Gluconate (CG) against *Acinetobacter* spp. studied in this work

Source	Isolate	Identification	MIC (ppm)	MBC (ppm)
Ready-to-eat salads	F3R18/7	*Acinetobacter baumannii*	31.2	31.2
	F3R13/1	*Acinetobacter baumannii*	31.2	31.2
	F1R13/6	*Acinetobacter baumannii*	31.2	31.2
	F2R21 *	*Acinetobacter baumannii*	250	250
	F2R13/7	*Acinetobacter baumannii*	31.2	31.2
	F4R15/7	*Acinetobacter nosocomialis*	31.2	31.2
	F4R15/6	*Acinetobacter nosocomialis*	62.5	62.5
	F3R12/7	*Acinetobacter nosocomialis*	15.6	15.6
	F4R15/3	*Acinetobacter nosocomialis*	62.5	62.5
	F1R13/7	*Acinetobacter nosocomialis*	62.5	62.5
	F5R14/3 *	*Acinetobacter gerneri*	31.2	31.2
Goat's raw milk	1708	*Acinetobacter guillauiae*	3.9	3.9
	1715	*Acinetobacter guillauiae*	7.8	7.8
	2017	*Acinetobacter ursingii*	3.9	7.8
	2008	*Acinetobacter ursingii*	7.8	7.8

*: Biofilm-producing isolate.

In 2014, Morrissey et al. published an extensive study proposing appropriate breakpoints for defining biocide resistance for triclosan, benzalkonium chloride, hypochloride, and CG, based on data from 3,327 clinical isolates. Although *Acinetobacter* spp. was not included in this study, the maximum epidemiological cut-off value to determine the tolerance of the microorganisms included in the study to CG is 64 ppm (Morrissey et al., 2014).

In general, *Acinetobacter* spp. isolated from the foods tested in this study tended to be susceptible to CG, with the MIC ranging from 3.9-62.5 ppm. The lowest MIC values (3.9-7.8 ppm) were obtained for the four isolates from raw goat milk. Most studies involving clinical isolates have shown low MIC values for CG against *Acinetobacter* spp., which generally range from 8-64 ppm (Kampf, 2016). The 11 isolates from the ready-to-eat salads included in this study presented MIC and MBC values of 15.8-62.6 ppm for CG. These higher values were comparable to those obtained for some clinical isolates including *A. baumannii*. Recently, a study was conducted in Israel, which showed that the MIC for CG in 17 *A. baumannii* isolates from clinical samples of hospitalized patients ranged from 8-64 µg/ml (8-64 ppm), with most isolates presenting the MIC of 16 or 64 ppm (Leshem et al., 2022). However, clinical isolates with the MIC for CG of up to 400 ppm have been reported. In a study involving 288 *Acinetobacter* spp. isolates in Japan, 28 (9.9%) presented reduced susceptibility to different disinfectants, 13 of which had MIC between 100-400 ppm for CG (Kawamura-Sato et al., 2010).

Of all 15 isolates included in this study, the isolate F2R21 presented the highest MIC and MBC values for CG (250 ppm). These values were comparable to those of clinical isolates with reduced susceptibility to CG and

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multidrug resistance (Kawamura-Sato et al., 2010). The reduced susceptibility could be attributed to previous exposure of the strains isolated from the ready-made salads to CG or other sanitizers at a different stage of the food production process. A similar phenomenon has been verified in *A. baumannii* isolates that showed reduced sensitivity to CG and benzalkonium chloride. The reduced sensitivity has been associated with resistance to other sanitizers, carbapenems, aminoglycosides, tetracycline, and ciprofloxacin (Fernández-Cuenca et al., 2015; Gadea et al., 2017). Notably, the isolate F2R21 is a biofilm producer. Microbial persistence due to biofilms in food processing environments can represents a challenge for food safety, as it is source of persistent or recurrent food contamination through microorganisms that are resistant to biocides and antibiotics (Oniciuc et al., 2019).

The most effective way to interrupt the transmission cycle of *Acinetobacter* spp. during food preparation and in the surrounding environment is by following the main control strategies that have been implemented in health care settings, including proper hand hygiene, cleaning the environment, and compliance with infection control measures (Cheng et al., 2015). Particularly, food care is a major factor in the success of these measures, since undercooked or raw foods can be potential carriers of *Acinetobacter* species. *A. baumannii* strains recovered from food are known to cause infections in the community and are also associated with nosocomial infections (Carvalheira et al., 2016, 2017).

Conclusion

To the best of our knowledge, this is the first study to assess the tolerance of CG in isolates of *Acinetobacter* spp. derived from food. Most of the studies described in literature were performed with clinical isolates, and MIC values for CG in *Acinetobacter* spp. are still debatable due to a lack of data. Our results showed that even food isolates can present MIC and MBC values for CG comparable to those of multidrug resistant isolates from clinical origin, suggesting that this sanitizer should be used sparingly for food handlers. Despite being effective, since residues of this biocide could cause an increase in the accumulation of antimicrobial-resistant bacteria such as *Acinetobacter* spp. through food, especially those consumed without any thermal treatment.

Author contributions

L.M.F. conducted the experimental work; L.M.F. and J.S.N. analyzed the data and wrote the manuscript. Both authors read and approved the final manuscript.

Conflicts of interest

There was no conflict of interest.

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