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Bacillus cereus Group Exhibits More Resistant to Chlorhexidine Rather Than *Bacillus subtilis* Group

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Abstract

Chlorhexidine is a widely used antiseptic agent; however, its bactericidal effect against bacterial endospores is limited. The genus *Bacillus* is spore-forming gram-positive bacteria that are ubiquitously found in the environment and cause opportunistic infection and food poisoning. The susceptibility of bacterial endospores to chlorhexidine was previously evaluated in *Bacillus subtilis*, but the primary target for disinfection with antiseptic agents, including chlorhexidine, should be harmful strains. We aimed to evaluate the susceptibility of harmful *cereus* group including *Bacillus cereus*, and to compare that with harmless *Bacillus* species, containing *B. subtilis*. We evaluated the susceptibility of the 15 strains of the *cereus* group to chlorhexidine in comparison with the 5 other strains, named the *subtilis* group in this study. Our results indicated that chlorhexidine exerted a bacteriostatic effect against *Bacillus* species at practical concentrations, especially during long-term exposure. The strains of *B. cereus* group in this study displayed relatively lower susceptibility to the antiseptic than the *B. subtilis* group according to the minimum inhibitory and bactericidal concentrations. We concluded that there are intrinsic differences in the susceptibility to chlorhexidine between the groups, but the molecular mechanisms are unknown. The minimum inhibitory or bactericidal concentrations of disinfectants other than chlorhexidine may also need to be clarified in the *B. cereus* and *B. subtilis* groups.

Keywords

Bacillus cereus, *Bacillus subtilis*, Chlorhexidine, Susceptibility

1. Introduction

Chlorhexidine is one of the most widely used biguanides for antiseptic purposes, such as skin surface preparation and intravascular catheter maintenance. Chlorhexidine inhibits bacterial growth by disrupting the structure of the cell membrane, leading to the leakage of cellular contents [1]. Chlorhexidine gluconate (CHG) or chlorhexidine acetate is generally used because of the low solubility of chlorhexidine base. Although the antiseptic activity of CHG has been validated in gram-positive and gram-negative bacteria and enveloped viruses, its efficacy against mycobacteria, nonenveloped viruses, fungi, and bacterial endospores is limited [2]. In spore-forming bacteria, chlorhexidine acts as a sporestatic agent rather than a sporicidal agent [3] [4] [5] [6]. Because CHG is generally ineffective against bacterial endospores, less attention has been paid to the differences in the action of CHG in the genus *Bacillus*. The efficacy of CHG against bacterial endospores was mainly examined using *B. subtilis*, the representative species of the genus. The bactericidal or inhibitory concentration of chlorhexidine in *Bacillus* spp. had not been studied extensively, because most antiseptic agents were generally ineffective against bacterial spores. *Bacillus* consists of spore-forming, facultative anaerobic or aerobic gram-positive bacilli, and *Bacillus* spp. is ubiquitously isolated from the environment, including the skin surface. The genus *Bacillus* comprises many species ranging from pathogenicity to animals, including *Bacillus cereus*, to harmless species, including *Bacillus subtilis*. Among them, the harmful species are classified into the *cereus* group, which comprises *B. cereus sensu stricto, Bacillus thuringiensis*, *Bacillus anthracis*, and other species *Bacillus weihenstephanensis*, *Bacillus mycoides*, and *Bacillus pseudomycoides* are also included in this group [7]. Several studies examined the activity of chlorhexidine against *B. subtilis*, whereas studies on the sensitivity of harmful species in the *B. cereus* group, which is the main target of disinfection, to CHG have been limited.

This study aimed to evaluate the susceptibility of the *B. cereus* group to CHG and compare it between other genus *Bacillus* species. In this study, we divided bacterial strains into two groups, the *cereus* group or *subtilis* group. The *cereus* group consists of representative strains of *B. cereus*, *B. thuringiensis*, and *B. weihenstephanensis*, and clinical isolates of *B. cereus*. The *subtilis* group consists of the *B. subtilis*, *Bacillus amyloliquefaciens*, and *Bacillus liqueniformis*. The bacteriostatic and bactericidal concentrations of CHG were compared among *Bacillus* spp. The decrease in bacterial counts under bacteriostatic concentrations was assessed by a time-kill assay using representative strains.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

The *Bacillus* strains employed in this study are listed in Table 1. The strains included 3 representative or genome strains of *B. cereus sensu stricto* (ATCC14579, type strain, ATCC10987, NC7401), and 10 clinical strains of *B.*

Table 1. Bacterial strains used in this study and MIC, MBC and disk diffusion test.

species	strain	MIC (mg/L)	MBC (mg/L)	Polymyxin B (mm)	Colistin (mm)	source	year	reference
cereus group								
Bacillus cereus	ATCC14579^T	3	10,000	9.1	-	milk spoilage	1887[a]	[10]
	ATCC10987	3	10,000	8.6	-	diarrheal food poisoning	1952[a]	[9] [11]
	NC7401	3	5,000	10.3	-	emetic food poisoning	1974[b]	[12] [13]
	BL6459	3	5,000	10.9	-	blood culture	2009[b]	
	BL6460	3	2,500	11.1	-	blood culture	2009[b]	
	TH119	3	10,000	9.3	-	blood culture	2010[b]	
	TH120	4	2,500	9.0	-	blood culture	2010[b]	
	STKT	3	5,000	10.3	-	blood culture	2007[b]	
	669601	3	2,500	11.4	-	blood culture	2011[b]	
	669602	3	10,000	10.7	-	blood culture	2011[b]	
	SUMK	3	2,500	10.5	-	blood culture	2007[b]	
	NC1241	3	2500	11.0	-	food spoilage	2012[b]	
	H27-5	4	5,000	11.1	-	skin surface of a patient	2015[b]	
Bacillus thuringiensis	NBRC101235^T	3	5,000	8.9	-	Tissue, animal	1946[a]	[8]
Bacillus weihenstephanensis	NBRC101238^T	3	5,000	9.9	-	pasteurized milk	1997[a]	[14]
subtilis group								
Bacillus subtilis	NBRC13719^T	1.5	2,500	13.7	9.9	unknown	1930[a]	[15] [16]
	PCI219	1.5	1,000	15.0	10.7	laboratory strain	1971[a]	[17]
Bacillus amyloliquefaciens	IFO3007	1.5	1,000	16.1	9.2	unknown	1946[c]	[18]
	IFO3025	1.5	1,000	15.5	9.5	unknown	1951[c]	[19]
Bacillus liqueniformis	NBRC14206	1.5	2,500	16.5	11.5	unknown	1982[c]	
other genus (control)								
Staphylococcus aureus	ATCC27664	1.5	50	10.8	-			
Escherichia coli	ATCC25922	1.5	50	16.2	12.6			

In the table, descriptive statistics are provided for the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of CHG, and the disk diffusion test was performed using polymyxin B and colistin. "T" in the "strain" column denotes the type strain. The sensitivity to polymyxin B or colistin was indicated by the zone of inhibition (mm), and "-" indicated no observation of growth inhibition around the antibiotic disk. The column "year" indicates the year that the strain was first described in the literature (a), separated from a source (b), or deposited in a bacterial culture collection (c).

cereus (BL6459, BL6460, TH119, TH120, STKT, 669601, 669602, SUMK, NC1241, and H27-5) [8]-[13]. For representative strains of other *cereus* groups, *B. thuringiensis* NBRC101235 (type strain) and *B. weihenstephanensis* NBRC101238 (type strain), were chosen [14]. *B. thuringiensis* NBRC101235 is listed as ATCC10792 in the American Type Culture Collection. *B. weihenstephanensis* NBRC101238 is the synonym of *B. mycoides*, and is listed as DSM11821 in Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. *B. subtilis sensu stricto B. subtilis subsp. subtilis* NBRC13719 and PCI219, *B. amyloliquefaciens* IFO3007 and IFO3025, and *B. licheniformis* NBRC14206 [15] [16] [17] [18] [19]. *E. coli* ATCC25922 and *S. aureus* ATCC27664 were used as references for gram-negative and gram-positive strains, respectively. All isolates were grown in MH broth (Becton, Dickinson and Company, Sparks, MD, USA), MH

agar, or standard nutrient agar (Atect Corp., Shiga, Japan) at 37˚C under atmospheric conditions. In this study, the counts of living bacterial cells were measured as the average colony-forming units (CFUs) on three MH agar plates.

2.2. Chemical Materials

CHG solution (20% w/v HIBITANE®, Sumitomo Dainippon Pharma Co., Ltd., Osaka, Japan) was used as a formulation of chlorhexidine. CHG was serially diluted with sterilized water in each concentration and used in sensitivity studies. For minimum bactericidal concentration (MBC) experiments, inactivation solution containing 3% Tween-80 (Sigma-Aldrich Co., St. Louis, MO, USA) and 0.3% lecithin (Fujifilm Wako Pure Chemical Co., Osaka, Japan) was prepared [20].

2.3. Determination of the MIC of CHG

The minimum inhibitory concentration (MIC) of CHG was assessed by the agar dilution method, referring to the method for dilution antimicrobial susceptibility tests of clinical and laboratory standard institute [21]. All tested bacterial strains were cultured overnight in MH broth, and suspensions were diluted in Dulbecco's phosphate-buffered saline (PBS, without calcium chloride and magnesium chloride, Sigma-Aldrich Co.) to McFarland 0.5 standard, equivalent to approximately 1×10^8 CFUs/mL. The diluted broths were added to MH agar plates containing CHG at a concentration of 0.5, 1, 1.5, 2, 2.5, 3, 4, or 5 mg/L. After incubation at 37˚C for 48 h, the minimum concentration at which bacterial growth was inhibited was determined as the MIC.

2.4. Determination of the MBC of CHG in Spores

Each strain was seeded onto a standard nutrient agar plate. After incubation overnight at 37˚C followed by 3 days at 25˚C days, the formation of endospores was confirmed via microscopic observation using the modified Wirtz-Conklin staining method referring to rapid staining techniques [22] [23]. The bacterial culture containing endospores was suspended in PBS to McFarland 0.5 standard. CHG was added at a final concentration of 50, 100, 250, 500, 1000, 2500, 5000, or 10,000 mg/L. After incubation at room temperature for 30 min, nine volumes of inactivation solution were added to the mixture to inactivate CHG. Then, 10 µL of bacterial suspension were placed on MH agar plates without CHG. After incubation at 37˚C for 48 h, the minimum concentration at which bacteria were killed was determined as the MBC. These experiments were repeated at least twice to confirm the results.

2.5. Time-Kill Test Using a Sub-Bactericidal Concentration of Chlorhexidine

The modified time-kill test was performed using eight *Bacillus* isolates to evaluate the changes of bacterial cell populations under bacteriostatic concentrations of chlorhexidine. Initially, 100 µL of an overnight bacterial culture was inoculated into 100 mL of fresh MH broth, and chlorhexidine solution was added

at a final concentration of 10 mg/L. The mixture in glass flasks was incubated at 37˚C with shaking at 160 rpm and recovered after 15 s, 10 min, 30 min, 1 h, 3 h, 6 h, 12 h, 24 h, or 48 h. The recovered mixture was seeded onto MH agar plates. After incubation at 37˚C overnight, the surviving bacterial population was calculated by counting colonies on the agar plate. The survival of bacterial cells was evaluated in comparison to the initial cell number. The experiments were repeated at least twice to confirm the results for each tested strain.

2.6. Microscopic Observation with CHG

Microscopic imaging was performed during incubation with several concentrations of CHG. Overnight cultures of *B. cereus* ATCC10987 and *B. amyloliquefaciens* IFO3007 were diluted in PBS to McFarland 0.5 standard and then incubated with 0, 10, 25, 50, or 100 mg/L CHG for 30 min at room temperature. One loop of the incubation mixture was placed on a slide glass, which was stained using the standard Gram staining method. After staining, the slides were examined using the oil immersion objective of an optical microscope (BX51 with DP73, Olympus, Tokyo, Japan).

2.7. Disk Diffusion Test Using Colistin and Polymyxin B

All isolates were subjected to a disk diffusion test using colistin (10 μg) and polymyxin B (300 μg), because the two antibiotics target bacterial cell membrane. A 0.5 McFarland standard suspension of the isolate was prepared and spread on an MH agar plate, and each antibiotic disk was placed on the plate. Plates were incubated at 37˚C for 20 h, and zone diameters (mm) were measured. The disk diffusion test was repeated at least two times, and the average diameter was calculated.

3. Results

3.1. Minimum Inhibitory Concentrations (MICs) of CHG

The MICs of CHG were higher in the *B. cereus* group than in the *B. subtilis* group (Table 1). The MICs of CHG ranged from 3 to 4 mg/L in the *B. cereus* group, which consisted of 13 strains of *B. cereus*, *B. thuringiensis* NBRC101235, and *B. weihenstephanensis* NBRC101238. Although chlorhexidine is generally considered more effective against gram-positive bacteria, the MICs of CHG were higher for the *B. cereus* group than for the gram-positive coccus *Staphylococcus aureus* ATCC27664. The MICs were also higher than that of the gram-negative strain *Escherichia coli* ATCC25922. It should be noted that the MICs of *S. aureus* and *E. coli* were both 1.5 mg/L in the present study.

In comparison, the MIC of CHG in the *B. subtilis* group was 1.5 mg/L, equivalent to that of the controls.

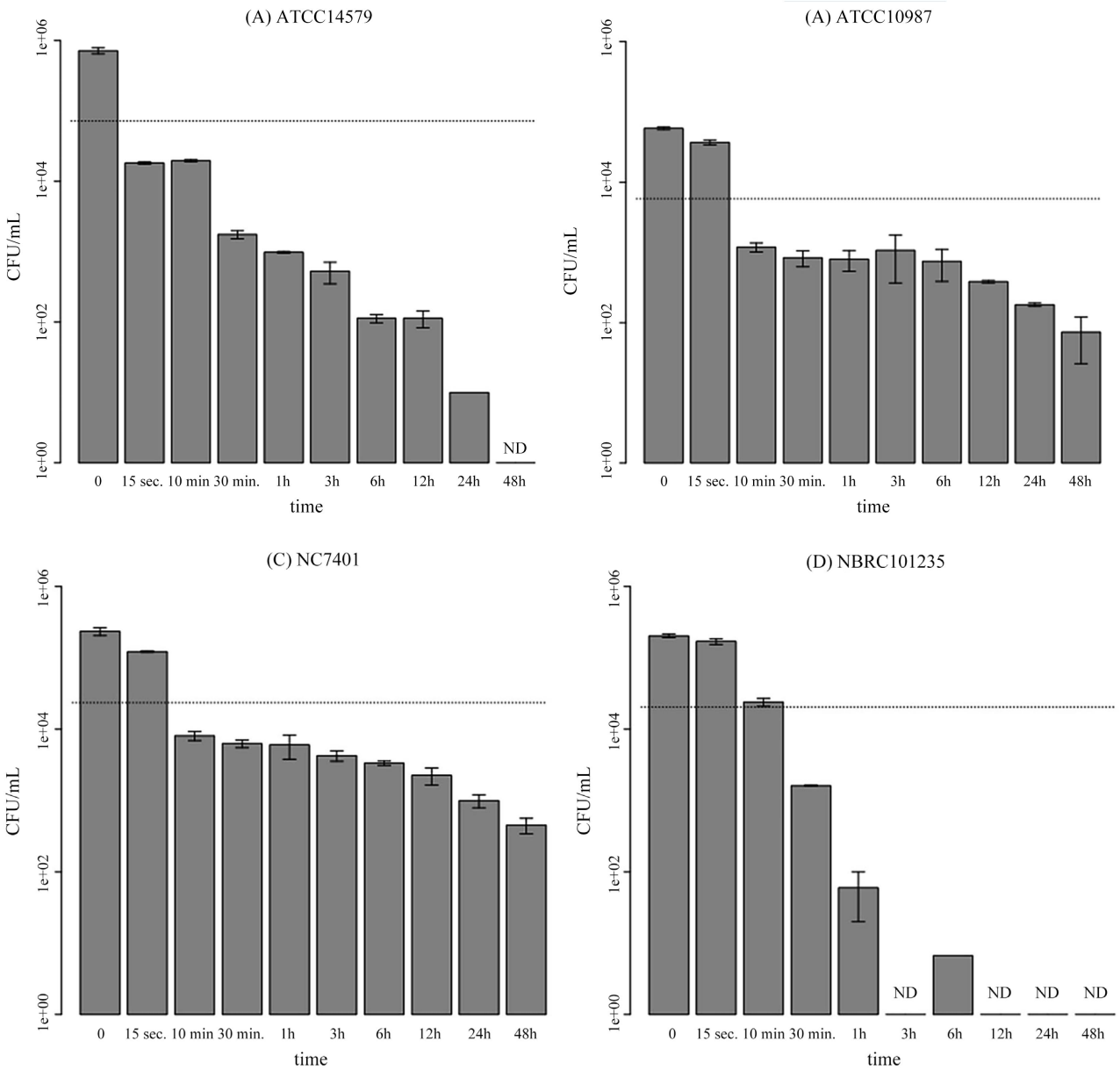
3.2. Minimum Bactericidal Concentrations (MBCs) of CHG for Spores of the Genus *Bacillus*

The MBCs of CHG were slightly higher for the *B. cereus* group than for the *B.*

subtilis group (Table 1). The MBCs of CHG ranged from 2,500 - 10,000 mg/L for the *B. cereus* group, versus 1,000 - 2,500 for the *B. subtilis* group. In both groups, the sporicidal concentration was nearly identical to the practically used concentration.

3.3. Time-Kill Assay of Chlorhexidine

The time-kill test was used to examine the survival ratio of *Bacillus* spp. under sub-bactericidal concentrations (Figure 1). In this study, all strain counts were decreased to below detectable levels after 48 h of incubation with 10 mg/L CHG in Mueller-Hinton (MH) broth. As shown in Figure 1, the counts of all tested strains decreased to 10% of the control level (MBC_{90}) within 30 min. There were no significant differences in effects between the *B. cereus* and *B. subtilis* groups.



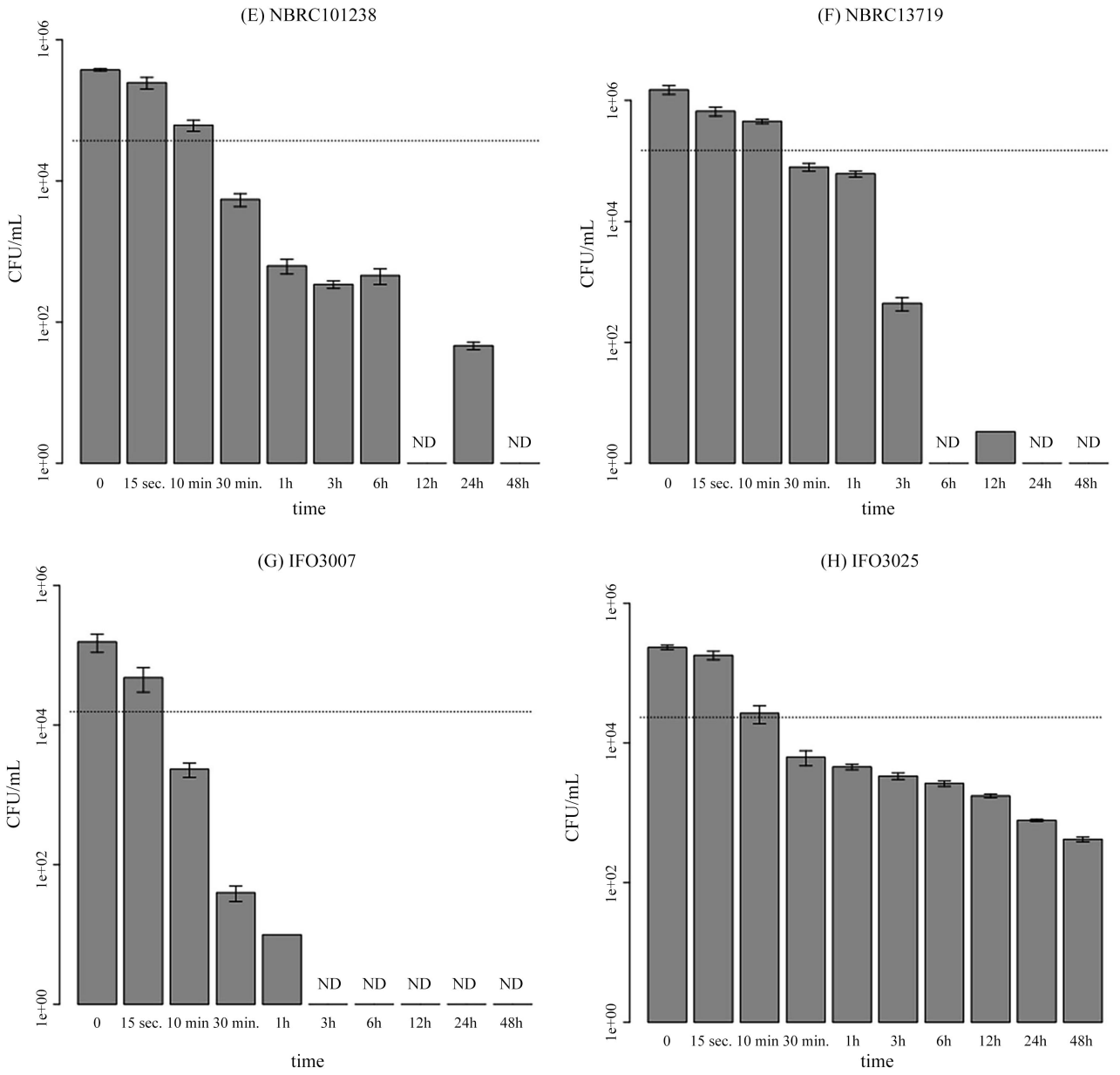


Figure 1. Bar graph of the time-kill test using a sub-bactericidal concentration (10 mg/L) of chlorhexidine. The time before the addition of chlorhexidine was set as time zero. Bar height represents mean survival cell number with colony forming unit per mL (CFU/mL). The bracket on the bars indicates the standard error (S.E.) of triplicate determinations in three experiments. In total, eight bacterial strains were examined; *B. cereus* ATCC14579 (A), *B. cereus* ATCC10987 (B), *B. cereus* ATCC10987 NC7401 (C), *B. thuringiensis* NBRC101235 (D), *B. weihenstephanensis* NBRC101238 (E), *B. subtilis* NBRC13719 (F), *B. amyloliquefaciens* IFO3007 (G), and *B. amyloliquefaciens* IFO3025 (H). Each *Bacillus* strain was incubated at 37˚C with shaking at 160 rpm with 10 mg/L chlorhexidine and recovered after 15 s, 10 min, 30 min, 1 h, 3 h, 6 h, 12 h, 24 h, and 48 h. The horizontal dot line in the graphs indicated the 10% survival rate (90% minimum bactericidal concentration; MBC_{90}) as a time zero for standard. ND indicated "not detected," meaning that no living bacteria were isolated the mixture.

3.4. Microscopic Observation

Cellular damage induced by CHG was assessed in both *B. amyloliquefaciens* IFO3025 and *B. cereus* ATCC10987 (Figure 2). Damaged cells and debris were observed in *B. amyloliquefaciens* IFO3025 incubated with 25 mg/L CHG (Figure

2(A)), whereas no cellular damage or debris were observed in *B. cereus* ATCC10987 at this concentration (Figure 2(B)). Meanwhile, cellular damage and debris were observed in *B. cereus* ATCC10987 exposed to 50 mg/L CHG (Figure 2(B)). This morphological observation supported the differences in the susceptibility to CHG between the *B. cereus* and *B. subtilis* groups. In a preliminary study, other strains belonging to the *cereus* group, *B. cereus* ATCC14579, NC7401, *B. thuringiensis* NBRC101235, and *B. weihenstephanensis* NBRC101238, were also observed to have cell damage similar to ATCC10987. Other strains belonging to the *subtilis* group, *B. subtilis* ATCC13719 and *B. amyloliquefaciens* IFO3007 in the *subtilis* group, were also observed to have similar cell damage *B. amyloliquefaciens* IFO3025 (data not shown).

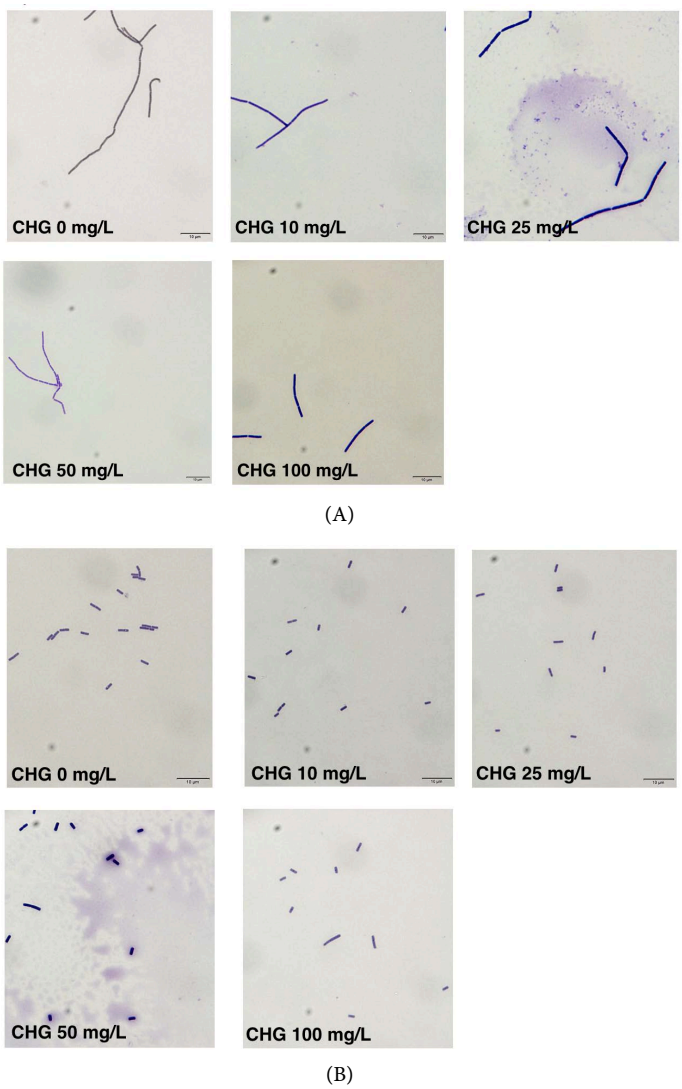


Figure 2. Microscopic observation of *B. amyloliquefaciens* IFO3025 (A) and *B. cereus* ATCC10987 (B). The pre-culture of the bacterial mixture was incubated with 10, 25, 50, or 100 mg/L chlorhexidine for 10 min and observed under the oil immersion objective of an optical microscope (×1000).

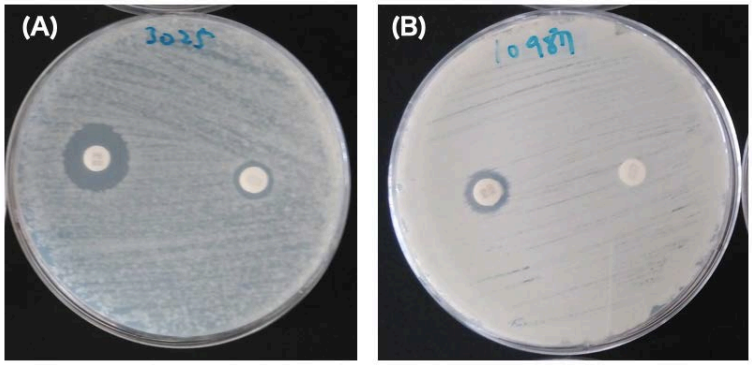


Figure 3. Disk diffusion test of colistin and polymixin B. The colistin and polymyxin disks were placed on which *B. amyloliquefaciens* IFO3025 (A) or *B. cereus* ATCC10987 (B) were grown. In both images, colistin disk was on the right side, and polymyxin B disk left side.

3.5. Disk Diffusion Test Using Colistin and Polymyxin B

All strains of *Bacillus* were tested with colistin and polymyxin disk on Muller-Hinton agar plate. Typical plate images of the growth inhibition zone around colistin and polymyxin disks were shown in **Figure 3**. Since no criteria have been set for drug resistance of polymyxins by the disk method, the diameters of inhibition zone were listed in **Table 1**. In the sensitivity for polymyxin B, the *cereus* group showed smaller inhibition zone (average 10.1 mm, S.D. ± 0.9) compared to that of *subtilis* group (average 15.3 mm, S.D. ± 1.0). In the colistin's sensitivity, the *cereus* group showed no inhibition zone, whereas the *subtilis* group showed an inhibition zone (average 10.1 mm, S.D. ± 0.8).

4. Discussion

In this study, the MICs of CHG were higher for the *B. cereus* group than for the *B. subtilis* group despite the use of concentrations below the practical preparation level. The Centers for Disease Control and Prevention found that 4% w/v chlorhexidine exhibits effective antiseptic activity [24]. In Japan, chlorhexidine formulations are usually used at concentrations of 0.02% - 0.5% (w/v) for skin surfaces, whereas these concentrations are contraindicated for exposure in the bladder, vagina, and ear mucosa. For these reasons, even though chlorhexidine does not sufficiently kill bacterial cells, including spores, the drug can be expected effectively to inhibit the growth of *Bacillus* spp. at practical concentrations. The MBC results supported prior findings that the endospores of *Bacillus spp.* are highly resistant to CHG [5] [6]. CHG functioned as a sporestatic agent rather than a sporicide for the endospores of *Bacillus* spp. One of the advantages of chlorhexidine is the long-term duration of its antiseptic effects. Although its short-term effects are inferior to those of other disinfectants such as glutaraldehyde, chlorhexidine is expected to be useful for disinfecting surfaces contaminated by *Bacillus* spp. because it disinfects while inhibiting growth over a long period.

Our result suggests the existence of intrinsic differences in the susceptibility to CHG between the *B. cereus* and *B. subtilis* groups. The MIC may be dependent on the bacterial species, strains, determination method, and chemical formulation. Shaker et al. reported that the inhibitory concentration of chlorhexidine acetate for *B. subtilis* NCTC8236 ranged from 0.4 to 4.75 mg/L depending on the methods and inoculum size [3] [5]. Cheung et al. reported the MIC of chlorhexidine, which was dissolved in MH broth containing 1% ethanol, for *B. subtilis* 60015 as 0.75 mg/L using the procedures recommended by the National Committee of Clinical Laboratory Standard [25]. However, few reports described the MICs of CHG in *Bacillus* spp. other than *B. subtilis*.

One hypothesis is that the differences in the susceptibility to CHG between bacterial groups were associated with adaptation. Increased usage of antiseptics clinically has promoted the acquisition of resistance to CHG in *Klebsiella pneumoniae* and *S. aureus* [26] [27]. Chlorhexidine was first developed in the 1950s, and its use has widely spread since the 1970s [28] Because the strains belonging to the *B. subtilis* group in this study were isolated in the pre-chlorhexidine era, it is necessary to survey the susceptibility to chlorhexidine in modern strains of the *B. subtilis* group. However, the standard strains, *i.e.*, *B. cereus* ATCC14579, ATCC10987, *B. thuringiensis* NBRC101235, and *B. weihenstephanensis* NBRC-101238, were also isolated in the pre-chlorhexidine era, and they were less sensitive to chlorhexidine than the *B. subtilis* group. Several strains of *B. cereus* isolated in the post-chlorhexidine era displayed lower susceptibility to CHG. For these reasons, we suggest that the differences in the susceptibility to CHG reflect intrinsic characteristics in the genus *Bacillus* rather than a chronological adaptation to chlorhexidine usage in humans.

The molecular mechanisms of the differences in chlorhexidine susceptibility between the examined *Bacillus* groups remain unclear. Previous studies identified the genes associated with chlorhexidine resistance. In *K. pneumoniae*, point mutations in *phoPQ* and *smvR*, which encode efflux pump regulators, have been linked to chlorhexidine resistance [27] [29]. In *S. aureus*, mutations in *norA*/*B*, which encodes an efflux pump, were suggested to be involved in resistance to chlorhexidine [26]. Interestingly, no gene homologous to *norA*/*B* was identified in *Bacillus* via a BLAST search [30]. However, many genes have been confirmed or hypothesized to encode multidrug efflux pumps in the *B. cereus* group [31]. Some efflux pumps may be involved in the sensitivity to chlorhexidine.

In general, chlorhexidine is more effective against gram-positive bacteria than against gram-negative bacteria. The negatively charged components of the cell wall and membrane, such as lipopolysaccharide, may act as permeability barriers, and they may be associated with the resistance to cationic antimicrobial agents in gram-negative bacteria [32]. The differential action of chlorhexidine was found in *E. coli* and *B. subtilis* [25]. The dented spots caused by chlorhexidine were localized to hemispherical caps in *B. subtilis*, whereas these spots were dispersed throughout the cell in *E. coli*. Several negatively charged phospholipids, such as cardiolipin and phosphatidylethanolamine, are localized on

hemispherical caps in *B. subtilis*. These phospholipids control membrane integrity during the division of vegetative cells or sporulation [33] [34]. It may be necessary to compare the state of localization and protection of negatively charged phospholipids to clarify the differences in the susceptibility to CHG between the *B. cereus* and *B. subtilis* groups.

Differences in sensitivity between the *B. cereus* and *B. subtilis* groups were also found in other polypeptide antibiotics that target the bacterial cell membrane. We evaluated the sensitivity to the polymyxins polymyxin B and colistin, also known as polymyxin E, via the disk diffusion method. Polymyxin B and colistin are synthesized by *Paenibacillus polymyxa*, previously known as *Bacillus polymyxa*. Polymyxin B is used as a component of selective media for *Bacillus* species, such as mannitol egg yolk polymyxin agar. Polymyxins are generally considered more useful for controlling gram-negative bacteria rather than gram-positive bacteria. Many species of *Bacillus* and related genera produce polypeptide antibiotics [35]. Drug resistance mechanisms may have developed during evolution in a niche to prevent self-poisoning by self-produced polypeptide antibiotics that target the cell membrane.

5. Conclusions

The susceptibility of genus *Bacillus*, mainly focused on the *cereus* group, to chlorhexidine was compared with that of the *subtilis* group. The results showed that the growth of the *cereus* group was inhibited at practical concentrations *in vitro*. However, the *cereus* group showed a low sensitivity to chlorhexidine rather than that of the *subtilis* group.

The limitation of this study is that the number of strains is quite a few, especially for harmless *subtlis* groups. Hence, it requires attention must be adapted in generalizing the results of this study to the entire genus *Bacillus*. However, despite the different eras, geographical locations, and species of the isolates showed the clear-cut differences between the *cereus* and *subtilis* groups in this study, especially in terms of MIC, suggest that there may be intrinsic differences between species in their susceptibility to chlorhexidine.

The effects of chlorhexidine on spore-forming bacteria have been described mainly for the *B. subtilis*. The main target of disinfection should not be harmless *B. subtilis*, but a group of harmful *cereus* group. We argue to the need for future evaluations of the effectiveness of disinfectants to be conducted on the *cereus* group.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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