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Effects of benzalkonium chloride adaptation on controlling *Listeria monocytogenes* biofilms and its growth in food

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Abstract: In this study the eradication effectiveness of four commonly used disinfectants against benzalkonium chloride (BC) adapted and non-adapted biofilms of *Listeria monocytogenes* was compared and the effects of food preservatives on the growth of these strains were comparatively evaluated on pasteurised chicken sausage. After BC adaptation, the minimum inhibitory concentrations (MICs) of BC against planktonic bacteria of *L. monocytogenes* increased, while the MICs of chlorine dioxide remained unchanged. BC adapted strains showed stronger biofilm formation than the wild-type parents. When used at 1 × MIC, the eradication rates of chlorine dioxide on biofilm biomass, cell viability and biofilm extracellular polymeric substance were higher than BC. When used at the recommended concentrations, chlorine dioxide exhibited the highest efficiency in BC adapted and non-adapted biofilm eradication. Among the four food preservatives, nisin showed the highest inhibition of both BC adapted and non-adapted strains grown on pasteurised chicken sausage. Our data suggest that proper use of BC is required to reduce the exposure of *L. monocytogenes* to sublethal concentrations of BC and the emergence of BC adapted strains.

Keywords: chlorine dioxide; bacterial biofilms; disinfectant adaptation; quaternany ammonium compounds

Listeria monocytogenes is a Gram-positive rod-shaped bacterium and major foodborne pathogen. Consumption of food contaminated by *L. monocytogenes* can cause sporadic and outbreak cases of listeriosis in humans (Macleod et al. 2022). *L. monocytogenes* has the ability to create robust biofilms on abiotic or biotic surfaces (Mazaheri et al. 2021). Extracellular polymeric substance (EPS) in biofilms acts as a protective barrier to prevent the entry of antimicrobials and disinfectants into *L. monocytogenes* cells, leading to the improvement of bacterial survival under environmental stress (Flemming et al. 2007). Therefore, control of *L. monocytogenes* biofilms remains a critical issue for food safety.

Disinfection is key in food processing facilities to reduce microorganisms to a level representing no risk to health. Chlorine-based disinfectants and quaternary ammonium compounds (QACs) are commonly used to disinfect food processing environments. Chlorine dioxide belongs to oxidative disinfectants and has the advantage of broad-spectrum bactericidal activity and high efficiency (Tomás-Callejas et al. 2012). Benzalkonium chloride (BC), representative of QACs, is a cationic surfactant with efficient bactericidal ability (Ortiz et al. 2014). Previous studies have reported that long-term exposure to sublethal concentrations of BC can promote resistance of *L. monocytogenes* to BC, which is known as BC adaptation (To et al. 2002; Lundén et al. 2003). BC adaptation of *L. monocytogenes* results in cross-adaptation to several antimicrobial agents and increased ability in biofilm formation (Romanova et al. 2006; Yu et al. 2018; Jiang et al. 2022). However, it is still uncertain whether BC adaptation of *L. monocytogenes* leads to cross-adaptation to chlorine dioxide. Recent studies have shown that chlorine dioxide can control bacterial

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biofilms, such as *Klebsilla* and *Pseudomonas* (Behnke and Camper 2012; Chai et al. 2014). However, the effects of chlorine dioxide on biofilms by *L. monocytogenes*, especially BC adapted strains of *L. monocytogenes*, have not been reported yet.

The direct addition of food preservatives is one of the ways to control microorganisms in food. Some food preservatives commonly used by food industry in China, such as nisin, ε-polylysine, potassium sorbate and sodium diacetate, have good antimicrobial activity against *L. monocytogenes* strains (Kaur et al. 2013; Lin et al. 2018). The risk of food contamination by BC adapted strains of *L. monocytogenes* in food processing environments cannot be ignored. It is unclear whether these commonly used food preservatives can effectively control the growth of BC adapted strains of *L. monocytogenes* in food.

The objectives of this study were to:

i) Investigate effects of BC and chlorine dioxide on mature biofilms;

ii) compare the eradication effectiveness of four commonly used disinfectants at recommended concentrations on *L. monocytogenes* biofilms;

iii) investigate the effects of four commonly used food preservatives on growth of BC adapted and non-adapted strains of *L. monocytogenes* on pasteurised chicken sausage.

MATERIAL AND METHODS

L. monocytogenes strains

Six strains of *L. monocytogenes* with different serotypes and their BC adapted strains (numbered with 'AT' after the wild-type strain designation) were used in this study (Table 1).

Table 1. *Listeria monocytogenes* strains used in this study

Strain	Source	Serotype	MIC (μg·mL^{-1})	
			BC	chlorine dioxide
LM2	raw pork	1/2c	2	10
LM2AT			12	10
LM45	raw chicken	1/2c	4	20
LM45AT			14	20
LM47	cooked meats	4b	6	20
LM47AT			12	20
LM61	raw pork	1/2c	2	20
LM61AT			12	20
LM70	raw beef	1/2b	6	20
LM70AT			12	20
LM83	cooked meats	1/2a	6	20
LM83AT			12	20

MIC – minimum inhibitory concentration; BC – benzalkonium chloride; AT – BC adapted strains

Disinfectants and food preservatives

BC was purchased from Aladdin (Shanghai, China), chlorine dioxide and peracetic acid from Habo Chemical Technology Co., Ltd. (Shanghai, China) and sodium diacetate (10% active chlorine) from Macklin (Shanghai, China). Nisin and ε-polylysine were purchased from Silver-Elephant Bio-engineering Co., Ltd. (China) and potassium sorbate (food-grade) and sodium diacetate (food-grade) from Beifang Xiaguang Food Additives Co., Ltd. (China).

Determination of minimum inhibitory concentrations (MICs) for BC and chlorine dioxide against planktonic bacteria

The MICs of two disinfectants for all *L. monocytogenes* strains were measured by the broth microdilution method (Yu et al. 2018). The concentrations of BC ranged from 2 to 18 μg·mL^{-1} with an increment of 2 μg·mL^{-1} and chlorine dioxide ranged from 5 to 40 μg·mL^{-1} with an increment of 5 μg·mL^{-1}.

Biofilm formation of *L. monocytogenes* by the crystal violet assay

Biofilms of all *L. monocytogenes* strains were quantified using the microplate method with crystal violet staining (Djordjevic et al. 2002). Briefly, *L. monocytogenes* cultures were transferred into the microtiter plates. Then the plates were incubated at 37 °C for 48 h in a static condition. Crystal violet dye (1%) was used to stain biofilms.

Biofilm assayed by confocal laser scanning microscopy (CLSM)

Biofilm formation assay by CLSM was conducted as described previously (Guilbaud et al. 2015). The commercial Live/Dead BacLight Bacterial viability kit (Molecular Probes, USA) was applied for biofilm stain. Images were acquired using confocal laser scanning microscope (TCS-SP8; Leica, Germany).

Quantitative detection of biofilm EPS

Biofilm EPS was extracted according to the method described previously (Liu et al. 2020; Wang et al. 2020).

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Table 2. Recommended concentrations of four disinfectants and their concentrations used in this study

Disinfectant	Recommended concentration (μg·mL^{-1})[a]	Concentration used in this study (μg·mL^{-1})
BC	200–1 000	200
Chlorine dioxide	50–100	50
Peracetic acid	500–1 000	500
Sodium hypochlorite	100–250	100

[a]recommended use concentrations of four disinfectants were provided by the National Standards of the People's Republic of China (General requirements for ordinary objects surface disinfectant, GB 27952-2020); BC – benzalkonium chloride

The eradication of mature biofilms by BC and chlorine dioxide

Three wild-type strains (L47, L70 and L83) and their BC adapted strains (L47AT, L70AT and L83AT) were used for the biofilm eradication by BC and chlorine dioxide. After washing the biofilms with sterile water, fresh brain heart infusion (BHI) broth supplemented with BC (1 × MIC; 6 μg·mL^{-1} for wild-type strains and 12 μg·mL^{-1} for BC adapted strains) or chlorine dioxide (1 × MIC; 20 μg·mL^{-1} for wild-type and BC adapted strains) was added to the wells and then incubated for 15 min at 37 °C.

Assessment of the eradication effectiveness of four commonly used disinfectants against *L. monocytogenes* biofilms

Table 2 shows the recommended use concentrations of four disinfectants in China and their concentrations used in our study. LM47 and LM47AT were selected for evaluating the biofilm eradication effectiveness of four disinfectants.

Effects of four food preservatives on growth of BC adapted and non-adapted strains on pasteurised chicken sausage

Pasteurised chicken sausage production. The frozen chicken breast (5 kg) was defrosted slowly at 4 °C and then ground into minced meat by a meat grinder (SY12-80; Shark Food Machinery Co., Ltd., China) and transferred into a chopper mixer (BZBJ-40; Aibo Technology Engineering Co., Ltd., China). The ingredients (60 g of salt, 25 g of potassium chloride, 45 g of glucose and 25 g of complex phosphate) dissolved in 900 mL of ice water were added and mixed with the minced meat. Then, the food preservative dissolved in 100 mL of ice water, carrageenan (20 g) and starch (200 g) were added successively. The mixture was pickled for 12 h at 4 °C and then poured into the casing using a sausage filler (ZY-15; Ruiheng Food Machinery, China). The sausage was heated in a water bath at 85 °C. After its core temperature reached 75 °C, the sausage was heated for another 10 min. The sausage was quickly cooled to room temperature, sliced, inoculated with *L. monocytogenes* and finally vacuum packaged. The weight of each vacuum packaged sample was approximately 25 g. Table 3 presents the maximum amount of each food preservative in cooked meat allowed and the concentrations used in this study.

Pasteurised chicken sausage inoculation. The samples were divided into two groups. One was inoculated with LM47 and the other with LM47AT. Each sample was inoculated on double surfaces with 250 μL of *L. monocytogenes* culture to achieve 4 log CFU·g^{-1}. Then the samples were vacuum packaged and stored at 4 °C.

Pasteurised chicken sausage sampling and L. monocytogenes enumeration. The following samples were taken for analysis: after *L. monocytogenes* in-

Table 3. The maximum amount of four food preservatives and their concentrations used in this study

Food preservative	The maximum amount (g·kg^{-1})[a]	Concentration used in this study (g·kg^{-1})
Nisin	0.50	0.20
ε-polylysine	0.25	0.20
Potassium sorbate	1.50	0.20
Sodium diacetate	3.00	0.20

[a]maximum amount of four food preservatives were provided by the National Standards of the People's Republic of China (Standard for the use of food additives, GB 2760-2014)

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oculation (day 0) and during storage on days 7, 14, 21, 28 and 35. Three samples from each treatment at each time point were transferred to the sterile bags containing 225 mL sterile saline and homogenised for 5 min. The homogenate was serially diluted in sterile saline, spread on *L. monocytogenes* selective agar and incubated at 37 °C for 48 h.

Statistical analysis

A two-tailed Student's test (SPSS version 20.0) was used for statistical analysis of the data. Results with a calculated *P* value below 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

MICs of BC and chlorine dioxide for *L. monocytogenes* strains

As shown in Table 1, each BC adapted strain had a higher BC MIC than its wild-type strain. However, BC adaptation did not alter the chlorine dioxide MICs of *L. monocytogenes* strains, suggesting that BC adaptation could not cause cross-adaptation to chlorine dioxide. Previous studies have reported that *L. monocytogenes* strains exhibit decreased sensitivity not only to BC but also to other antimicrobial agents, such as ethidium bromide, and cephalosporins after BC adaptation (Romanova et al. 2006; Yu et al. 2018). Cross-adaptation to these agents is possibly due to the involvement of a common mechanism. For example, BC and ethidium bromide are found to be substrates for the same efflux pump multiple drug resistance (MdrL) in *L. monocytogenes* (Romanova et al. 2006). Overexpression of MdrL by BC adaptation increases efflux for pumping BC and ethidium bromide, leading to cross-adaptation of the two agents (Romanova et al. 2006; Yu et al. 2018). In this study, cross-adaptation was not observed between BC and chlorine dioxide, indicating different mechanisms of action of the agent on *L. monocytogenes*.

Biofilm formation of *L. monocytogenes* strains

Biofilm biomass of all BC adapted strains was significantly higher ($P < 0.05$) than that of their corresponding wild-type strains (Figure 1A). CLSM images showed that biofilms of BC adapted strain were denser and thicker than the wild-type, which was consistent with results from biofilm biomass determination (Figure 1B). These data indicate that adaptation to BC improves *L. monocytogenes* biofilm formation. Strains with the same BC MICs exhibited different biofilm-forming abilities, suggesting no correlation between BC sensitivity and biofilm formation ability in *L. monocytogenes*. Efflux pumps are always considered as an important mechanism for BC resistance (Romanova et al. 2006; Yu et al. 2018). Biofilm formation of *L. monocytogenes* is influenced by many factors, such as flagella, EPS and quorum sensing

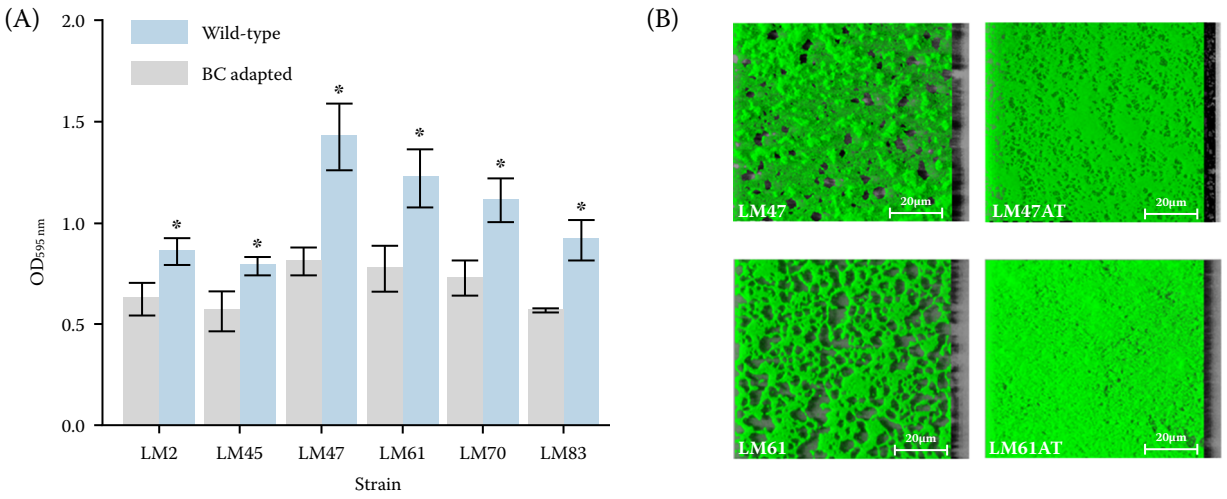

Figure 1. (A) Biofilm biomass of the wild-type and banzalkonium chlorine (BC) adapted strains determined by the microplate method with crystal violet staining; (B) confocal laser scanning microscopy (CLSM) images of two wild-type strains (LM47 and LM61) and their BC adapted strains (LM47AT and LM61AT)

Biofilms of all *Listeria monocytogenes* strains were incubated at 37 °C for 48 h; the asterisk indicates a significant difference between the BC adapted strain and its wild type

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system (Ortiz et al. 2014; Jiang et al. 2022). Since the mechanisms behind BC resistance and biofilm formation in *L. monocytogenes* are different, it is not surprising that strains with the same BC MICs exhibit different biofilm formation abilities.

EPS content in biofilms. Results of extracellular polysaccharide, extracellular protein and extracellular DNA in *L. monocytogenes* biofilms are presented in Figure 2A, B and C, respectively. Except for LM45AT and LM47AT, the extracellular polysaccharide content of other BC adapted strains was significantly higher ($P < 0.05$) than that of the corresponding wild-type strains. All BC adapted strains showed significantly increased ($P < 0.05$) extracellular protein in relation to the corresponding wild-type strains. Only the strain LM47AT showed a significant increase of 18.9% ($P < 0.05$) in extracellular DNA compared with its parent strain. Increased extracellular DNA content was also observed in the rest BC adapted strains, but there was no statistically significant difference ($P > 0.05$). As EPS contribute to cell adhesion and facilitate biofilm formation (Flemming et al. 2007), secreting more EPS is one of the reasons that BC adapted strains exhibit higher ability to form biofilms.

Eradication of mature biofilms by BC and chlorine dioxide

As shown in Figure 3A, the eradication rates of BC (1 × MIC) on biofilms of the BC adapted strains were much lower ($P < 0.05$) than those of their parent strains. The MICs for three BC adapted strains were 12 μg·mL^{-1} and BC MICs of their parent strains were 6 μg·mL^{-1}. For example, BC at 6 μg·mL^{-1} eliminated 47.2% of LM47 biofilms, while BC at 12 μg·mL^{-1} eliminated only 34.1% biofilms of LM47AT. Higher concentration of BC did not improve the biofilm eradication rate of BC adapted strains, suggesting that BC adaptation increases the difficulty of biofilm eradication by BC. BC adapted strains produced more EPS and exhibited higher ability to form biofilms compared to the wild type strains, which may result in dif-

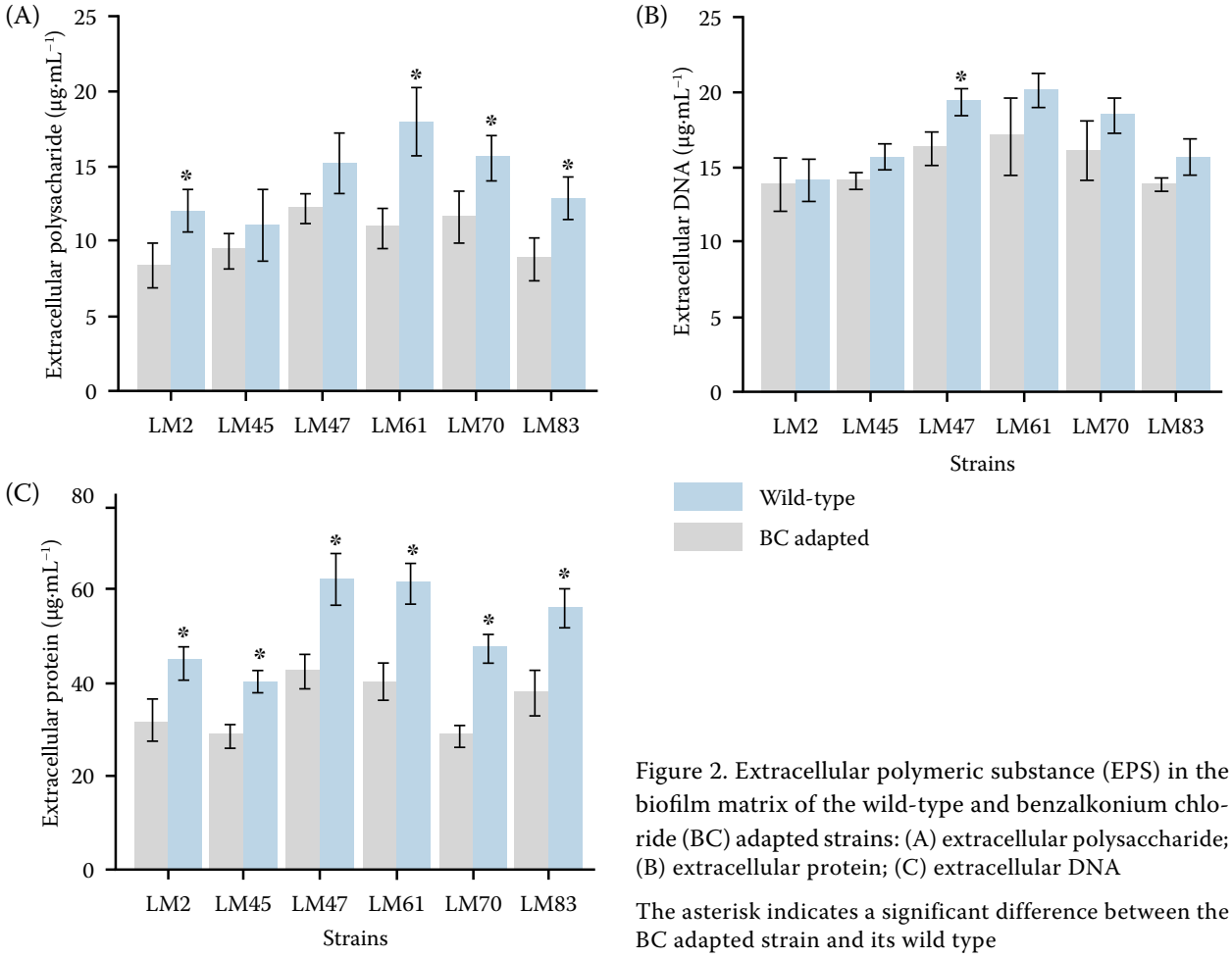

Figure 2. Extracellular polymeric substance (EPS) in the biofilm matrix of the wild-type and benzalkonium chloride (BC) adapted strains: (A) extracellular polysaccharide; (B) extracellular protein; (C) extracellular DNA

The asterisk indicates a significant difference between the BC adapted strain and its wild type

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ficulty in eliminating biofilms. However, no significant difference ($P > 0.05$) in biofilm eradication rates was observed between BC adapted and their corresponding wild-type strains when treated with chlorine dioxide at 1 × MIC, suggesting that BC adaptation has no effect on the eradication effectiveness of chlorine dioxide against *L. monocytogenes* biofilms. When the concentration of two disinfectants was 1 × MIC, chlorine dioxide showed higher biofilm eradication rates than BC, regardless of whether the strain was wild type or BC adapted.

As shown in Figure 3B, the killing rates of BC (1× MIC) on biofilm bacteria of the wild-type strains were higher ($P < 0.05$) than their corresponding BC adapted strains. For all strains, the eradication rates of cell viability in biofilms by 1 × MIC of chlorine dioxide were significantly higher ($P < 0.05$) than those by 1 × MIC of BC.

As shown in Figure 4, the eradication rates of chlorine dioxide (1 × MIC) on extracellular polysaccharide, extracellular protein and extracellular DNA of all strains were significantly higher ($P < 0.05$) than those of BC (1 × MIC).

Disinfectants BC and chlorine dioxide not only kill planktonic bacteria but also eradicate biofilms by foodborne pathogens (Chai et al. 2014; Rodríguez-Melcón et al. 2019). Rodríguez-Melcón et al. (2019) found that BC could remove most biofilm biomass but reduce cell viability in biofilms by *L. monocytogenes* only to a lesser extent. Given that effects of BC on mature biofilms formed by different strains may be related to the characteristics of strains and only a few strains were included in our study, it was difficult to compare our results with the previous ones. When the concentration of two disinfectants was 1 × MIC, the eradication rates of chlorine dioxide on biofilm biomass, cell viability and biofilm EPS were higher than those of BC.

Comparison among the eradication effectiveness of four disinfectants against *L. monocytogenes* biofilms

Effects of disinfectants on biofilm biomass and cell viability in biofilms of BC adapted and non-adapted strains are presented in Figure 5. Chlorine dioxide exhibited the highest eradication rate against non-adapted strain LM47 biofilm biomass and viable bacteria in biofilm, followed by BC, peracetic acid and sodium hypochlorite. Similar results were observed for BC adapted strain LM47AT.

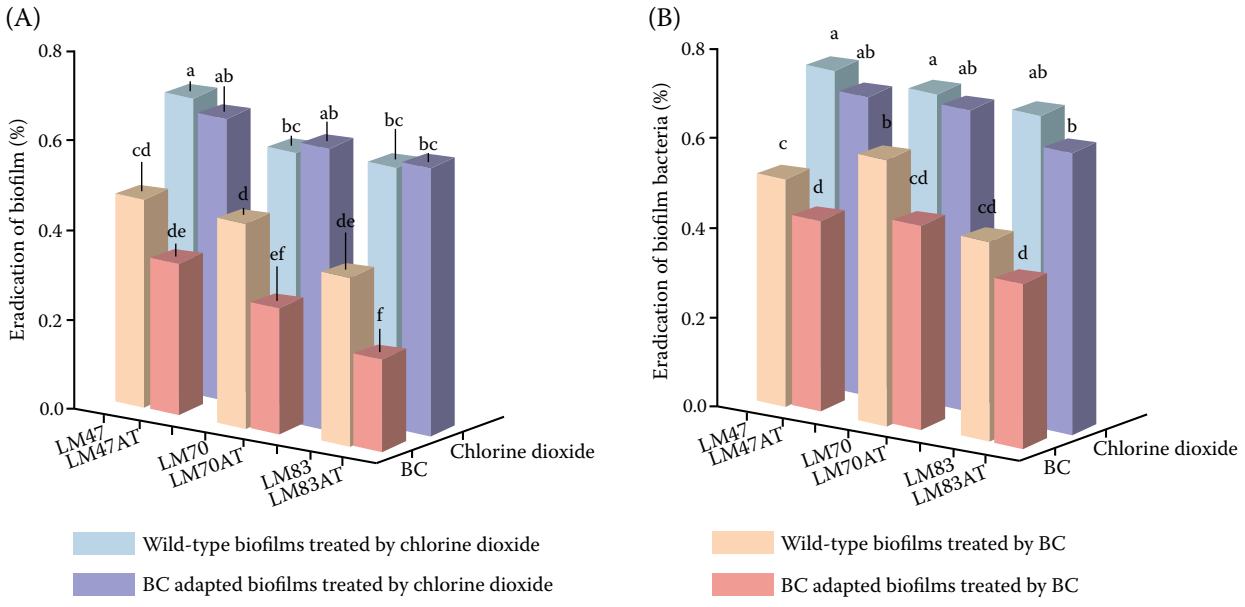

Figure 3. Effects of benzalkonium chloride (BC) and chlorine dioxide on preformed biofilms of the wild-type and BC adapted strains of *Listeria monocytogenes*: (A) biofilm biomass; (B) surviving cells in biofilms

The BC minimum inhibitory concentrations (MICs) of LM47, LM70 and LM83 were 6 μg·mL^{-1} and the BC MICs of LM47AT, LM70AT and LM83AT were 12 μg·mL^{-1}; the MICs of chlorine dioxide for three wild-type strains and their BC adapted strains were 20 μg·mL^{-1}; the mature biofilms were treated with BC (6 μg·mL^{-1} for wild-type strains and 12 μg·mL^{-1} for BC adapted strains) or chlorine dioxide (20 μg·mL^{-1}) for 15 min at 37 °C; different letters indicate significant differences at $P < 0.05$

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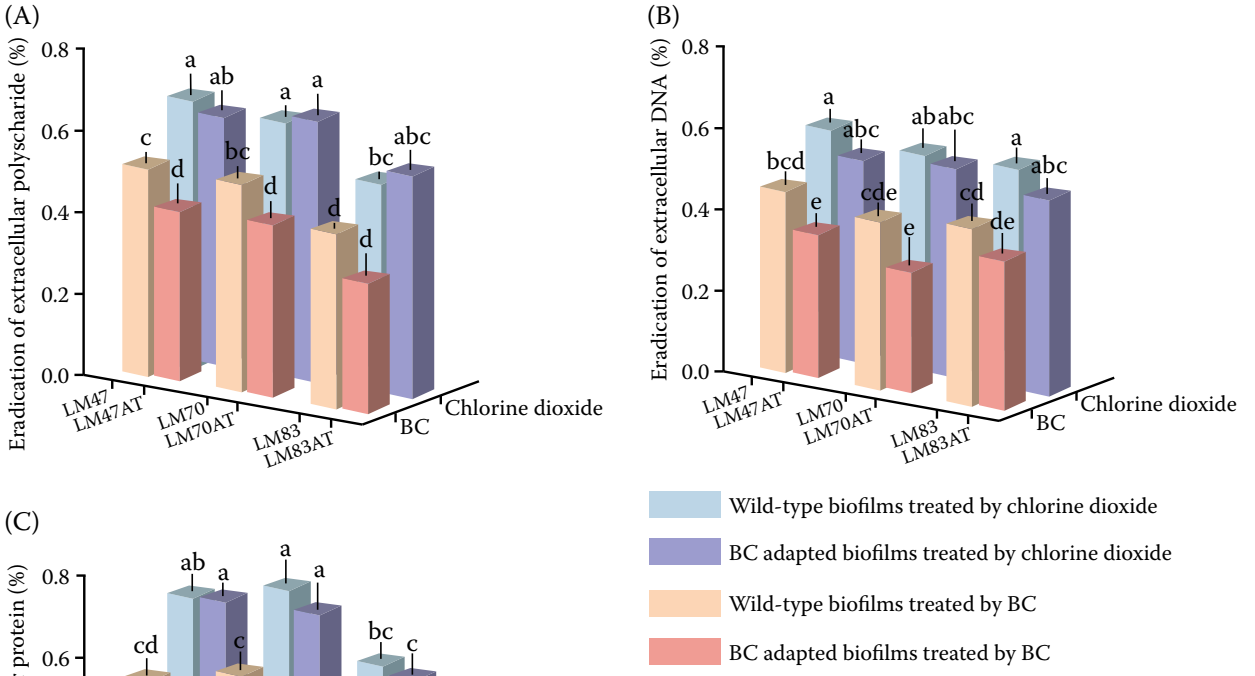

Figure 4. Effects of benzalkonium chloride (BC) (1 × minimum inhibitory concentration (MIC); 6 μg·mL^{-1} for wild-type strains and 12 μg·mL^{-1} for BC adapted strains) and chlorine dioxide (1 × MIC; 20 μg·mL^{-1}) on extracellular polymeric substance (EPS) in mature biofilms of the wild-type and BC adapted strains of *L. monocytogenes*: (A) extracellular polysaccharide; (B) extracellular protein; (C) extracellular DNA

different letters indicate significant differences at $P < 0.05$

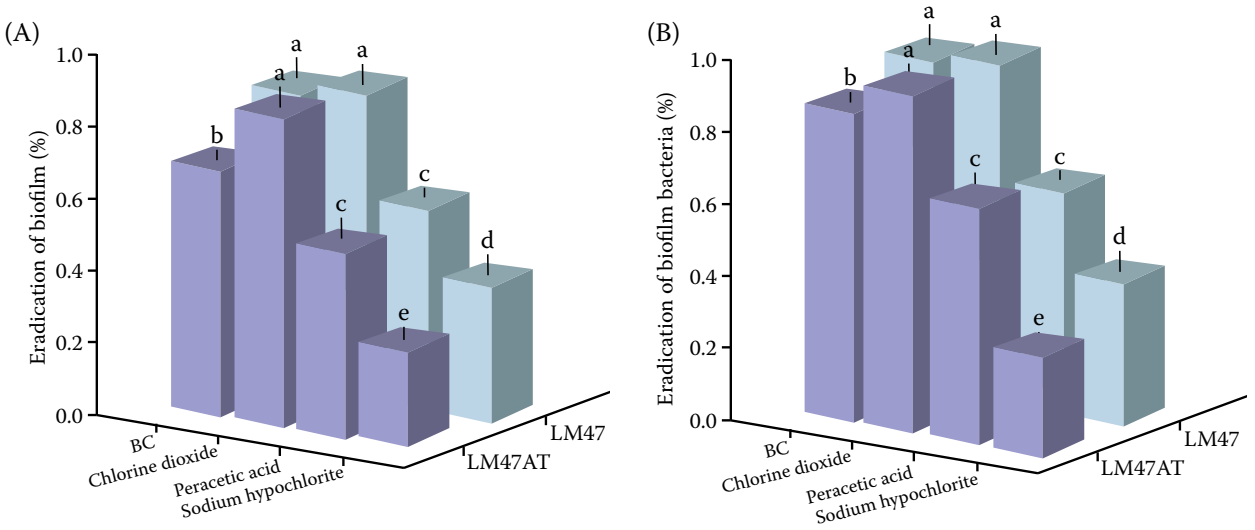

Figure 5. Effects of four disinfectants on mature biofilms of LM47 and LM47AT: (A) biofilm biomass; (B) surviving cells in biofilms

BC – benzalkonium chloride; after incubation at 37 °C for 48 h, the mature biofilms were exposed to disinfectants for 15 min, then the eradication of biofilm biomass and viable bacteria in biofilms was detected; different letters indicate significant differences at $P < 0.05$

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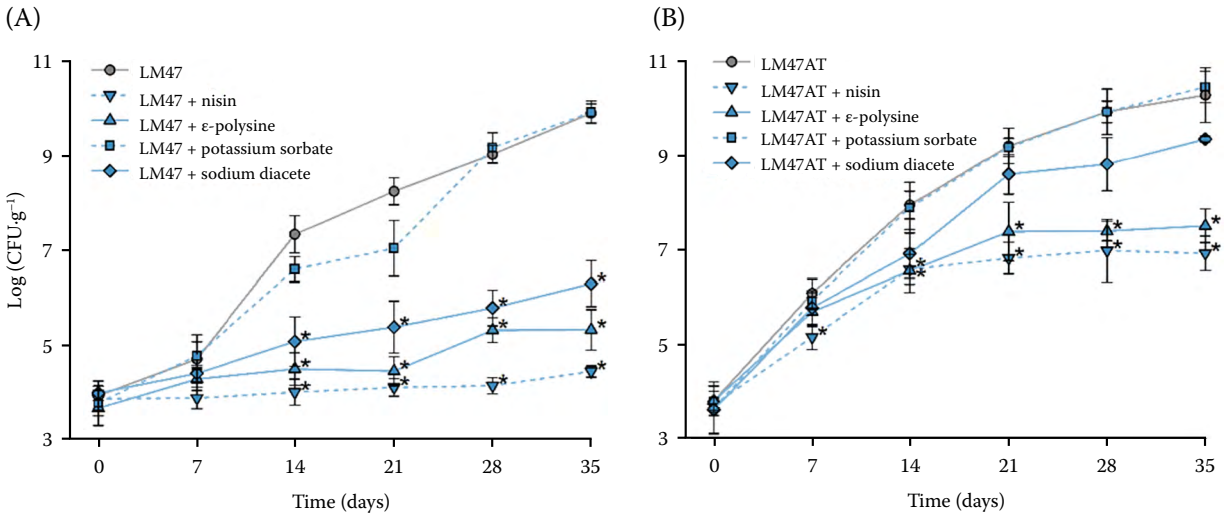

Figure 6. Effects of four food preservatives on growth of (A) LM47 and (B) LM47AT on pasteurised chicken sausage
The asterisk indicates a significant difference between the food preservative treatments and the control (grey solid circle)

The eradication rates of BC and sodium hypochlorite on BC adapted biofilms were significantly lower than those on non-adapted biofilms. However, no significant difference in biofilm eradication rates of chlorine dioxide and peracetic acid was observed between BC adapted and non-adapted strains. It was speculated that the eradication effectiveness of BC and sodium hypochlorite against *L. monocytogenes* biofilms may be affected by BC adaptation, given that BC adapted strains showed a higher ability to form biofilms than non-adapted strains. EPS in biofilms can impede the penetration of disinfectants. As BC and sodium hypochlorite have weak permeability to EPS (Ortiz et al. 2014; Rodríguez-Melcón et al. 2019), more EPS produced by BC adapted strains increased the difficulty of these two disinfectants to penetrate into EPS, leading to lower eradication rates of BC and sodium hypochlorite on BC adapted biofilms. Adaptive response to BC led to poor disinfection of this disinfectant on *L. monocytogenes* biofilms. Chlorine dioxide exhibited excellent biofilm eradication ability at low concentrations, and its eradication effectiveness was not influenced by BC adaption. Thus, it is necessary to rotate disinfectant agents such as the rotation of BC and chlorine dioxide in order to effectively remove *L. monocytogenes* biofilms in food processing environments.

Control of BC adapted and non-adapted *L. monocytogenes* on pasteurised chicken sausage by four food preservatives

As shown in Figure 6A, nisin had the best inhibitory effect on the growth of LM47 on pasteurised chicken sausage, followed by ε-polylysine, sodium diacetate and potassium sorbate. As shown in Figure 6B, nisin exhibited higher ability than ε-polylysine to inhibit the growth of LM47AT. Although LM47AT counts in sodium diacetate treatment were lower than the group at each time point, there was no significant difference ($P > 0.05$). LM47AT counts in potassium sorbate treatment at each time point were very close to those of the control. All preservatives tested in this study had lower abilities to inhibit the growth of LM47AT than LM47 on pasteurised chicken sausage.

An interesting phenomenon was observed when compared with two control groups: LM47 counts increased sharply at day 14, while LM47AT at day 7. This suggests that the lag-phase duration of BC adapted strain was shorter than that of non-adapted strain. BC adaptation may cause genetic variations, which leads to changes in growth characteristics of BC adapted strains. Thus, the difference in the lag-phase duration between L47 and L47AT may be due to genetic factors. Moreover, LM47AT counts were higher than those of LM47 at each time point, indicating that BC adapted strain showed better growth than non-adapted strain on pasteurised chicken sausage. Among the four food preservatives, nisin showed the highest inhibition of both BC adapted and non-adapted strains. It was worth noting that the inhibitory effect of four preservatives on BC adapted strain was lower than that on non-adapted strain, suggesting that BC adaptation increased the difficulty of controlling the growth of *L. monocytogenes* in food

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with preservatives. Thus, it is important to use the disinfectant BC according to standardised protocols to reduce the exposure of *L. monocytogenes* to sublethal concentrations of BC and the emergence of BC adapted strains.

CONCLUSION

In summary, BC adapted strains had a higher ability to form biofilms with more EPS than their corresponding wild-type strains. The eradication effectiveness of chlorine dioxide was better than that of BC when their concentrations were 1 × MIC. When used at the recommended concentrations, chlorine dioxide exhibited the highest efficiency in BC adapted and non-adapted biofilm eradication. BC adapted strain showed better growth than non-adapted strain on pasteurised chicken sausage. Among the four food preservatives, nisin showed the highest inhibition of both BC adapted and non-adapted strains. Our results suggest that BC adaptation increases the difficulty of controlling *L. monocytogenes* biofilm by disinfectants and its growth in food with preservatives, posing a potential threat to food safety.

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