https://doi.org/10.17221/44/2024-CJFS

Effect of the disinfectant benzalkonium chloride on *Listeria monocytogenes* biofilm

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Citation: Yang C., Zhang X., Yu T., Wang L., Zhu Z., Fan X. (2024): Effect of the disinfectant benzalkonium chloride on *Listeria monocytogenes* biofilm. Czech J. Food Sci., 42: 295–304.

Abstract: *Listeria monocytogenes* is capable of forming biofilms on the food contact surfaces, increasing the risk of food contamination by this pathogen. The disinfectant benzalkonium chloride (BC) is commonly used to control *L. monocytogenes* in the food industry. This study aimed to investigate effects of BC on *L. monocytogenes* biofilms. Biofilm biomass was measured by the microplate method with crystal violet staining. Results from the broth microdilution method showed that the minimum inhibitory concentration (*MIC*) of BC against *L. monocytogenes* 10403S was 8 μg·mL^{-1}. Sub-*MIC*s of BC inhibited the biofilm formation and lethal concentrations of BC removed mature biofilms of *L. monocytogenes* 10403S. The presence of BC reduced extracellular proteins and exopolysaccharides in biofilms. Additionally, upregulation of quorum sensing gene *luxS* and *agrBDCA* and downregulation of flagellum motility genes *flaA*, *motA*, and *motB* were observed in the presence of BC. The BC disinfectant has an excellent anti-biofilm activity against *L. monocytogenes*.

Keywords: foodborne pathogen; disinfection; anti-biofilm; extracellular polymeric substances

Listeria monocytogenes, a Gram-positive bacterium, is recognised as an important foodborne pathogen. This bacterium can cause listeriosis, a severe invasive infection with high mortality rates in humans (Brusa et al. 2021). Food contaminated by *L. monocytogenes* is recognised as the main vehicle for acquiring listerial infections (Churklam et al. 2020).

L. monocytogenes can adhere and form biofilms on various food contact surfaces, which enhances its ability to persist in food-processing environments for a long time (Jiang et al. 2022). Extracellular polymeric substances (EPS) in biofilm protect *L. monocytogenes* from antimicrobials, which helps this bacterium cope with adverse environments (Gautam et al. 2022). *L. monocytogenes* biofilms represent potential sources of food contamination. Therefore, controlling *L. monocytogenes* biofilms is one of the most important issues in the food industry.

Disinfectants are commonly used in the food industry to avoid the microbiological contamination of food products and reduce the risk of foodborne diseases. Quaternary ammonium compounds (QACs) are widely found in disinfectants used in food-processing environments. Benzalkonium chloride (BC) is one of the most commonly studied QAC disinfectants, and it exhibits good antibacterial activity against many important foodborne pathogens, including *L. monocytogenes* (Martínez-Suárez et al. 2016). Many previous studies have reported BC susceptibility of *L. monocytogenes* planktonic cells (Bonneville et al. 2020; Rodríguez-Melcón et al. 2023). However, data on the effects of BC on *L. monocytogenes* biofilms are scarce.

Therefore, the aim of this study was to investigate effects of BC on biofilm formation, mature biofilms, EPS production in biofilms, as well as expression of genes for biofilm formation in *L. monocytogenes*.

https://doi.org/10.17221/44/2024-CJFS

MATERIAL AND METHODS

Bacterial strains and growth conditions. *L. monocytogenes* 10403S, EGD-e, and three previously characterised *L. monocytogenes* strains (HL12, HL38, and HL91) were used in this study (Table 1). Minimum inhibitory concentrations (*MIC*s) of BC for HL12, HL38, and HL91 were reported in our previous study (Jiang et al. 2016). The *qac* genes and *bcrABC* are responsible for BC resistance in *L. monocytogenes*. However, neither *qac* genes nor the *bcrABC* gene cassette was present among the five strains tested (data not shown). *L. monocytogenes* 10403S was used for growth curve, killing curve, biofilm assay and biofilm EPS determination at different concentrations of BC because its phenotypic and genotypic characteristics are well documented and it has excellent ability to form biofilms. *L. monocytogenes* EGD-e, HL12, HL38, and HL91 were used for evaluating the effects of BC on bacterial biofilms at a certain concentration. All *L. monocytogenes* strains were grown in brain heart infusion (BHI; Huankai Ltd., China) broth at 37 °C.

Determination of minimum inhibitory concentration (*MIC*). The *MIC* for BC against *L. monocytogenes* 10403S and EGD-e was determined by the broth microdilution method as described previously (Bonneville et al. 2020). In brief, strains were incubated in BHI broth with different concentrations of BC using 96-well microtiter plates, with an inoculum of 10^4 to 10^5 CFU·mL^{-1} (CFU – colony forming unit). The plates were incubated at 37 °C for 24 h. Growth was recorded by measuring optical density $OD_{630\,nm}$ using a microplate reader (Sanco Instrument, China).

Growth curve analysis. The growth curve of *L. monocytogenes* 10403S was constructed as previously described (Jiang et al. 2019). Overnight cultures of *L. monocytogenes* 10403S were diluted in fresh BHI broth at the ratio of 1:100. Three hundred microlitres of diluted cultures were transferred to the wells of the honeycomb plate. The strain was grown in a Bioscreen C microbiology reader (Finland) for 48 h at 37 °C and the $OD_{600\,nm}$ was measured at 15-min intervals. To investigate the effect of BC on *L. monocytogenes* growth, different concentrations of BC (1, 2, and 4 μg·mL^{-1}) were added to BHI broth.

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

Strain	Source	Serotype	*MIC* of BC (μg·mL^{-1})
10403S	human	1/2a	8
EGD-e	human	1/2a	6
HL12	faw chicken	1/2b	6
HL38	raw chicken	1/2a	4
HL91	raw pork	1/2a	12

MIC – minimum inhibitory concentration; BC – benzalkonium chloride

Killing curve. The killing curves by BC in *L. monocytogenes* 10403S were determined as previously described (Jiang et al. 2019). Bacterial cultures in the logarithmic phase ($OD_{600\,nm}$ of 0.6) were centrifuged and the supernatant was discarded. After washing, the precipitate was resuspended in BHI with BC at 16 μg·mL^{-1} and 32 μg·mL^{-1}, respectively. During a 30 min exposure time, colony counting was performed by the spread plate method at 10-min intervals.

Effect of benzalkonium chloride on *Listeria monocytogenes* biofilm formation. *L. monocytogenes* biofilm was incubated in 96-well plates as previously described (Jiang et al. 2022). Overnight cultures of *L. monocytogenes* were diluted (1:100) in fresh BHI broth. Two hundred microlitres of cultures were transferred into wells of the plate and then the plate was incubated at 37 °C for 48 h. To determine the growth of planktonic cells, 100 μL of the bacterial cultures were centrifuged and the pellets were resuspended in 1 mL of sterile saline. The bacterial cultures were 10-fold serially diluted and 100 μL volumes were taken for the colony count. To assess biofilm formation, the medium containing planktonic cells was removed and then the biofilms in wells were stained with 1% crystal violet (200 μL) and finally the absorbance was read at 595 nm. To investigate the effects of BC on *L. monocytogenes* biofilm formation, different concentrations of BC (1, 2, and 4 μg·mL^{-1}) were added to BHI broth and the biofilms were incubated at 37 °C for 48 h. The biofilms were visualised under a DMi1 inverted optical microscope (Leica Microsystems, Germany).

Effect of benzalkonium chloride on *Listeria monocytogenes* mature biofilms. After 48-h incubation, the medium was removed and fresh BHI broth with different concentrations of BC (8, 16, and 32 μg·mL^{-1}) was added to the wells and incubated for 30 min. The biofilm biomass was quantified by crystal violet staining as described above. To quantify the biofilm bacteria, the wells were washed with sterile water. And then 200 μL of sterile saline were added to each well and its surface was thoroughly scratched with a plastic pipette tip. Recovered biomasses were vortexed and then the bacterial cultures were spread onto BHI plates for counting the colonies.

Quantification of biofilm extracellular polymeric substances. The EPS in *L. monocytogenes* biofilm

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were extracted as previously described (Liu et al. 2020; Wang et al. 2020). The biofilms were washed and redissolved with sterile phosphate-buffered saline (PBS) and sonicated at 50 kHz for 5 min. The mixture was centrifuged at 8 000 g for 30 min at 4 °C and the supernatants were filtered through a 0.22 μm membrane filter. The extracellular polysaccharide was quantified using the phenol-sulphuric acid method with glucose as the standard. Two millilitres of the EPS sample were transferred into a centrifuge tube. One millilitre of the phenol solution (50 g·L^{-1}) was added and mixed for 15 s. Then 5 mL of sulphuric acid (95%) were added and incubated in dark for 10 min and the $OD_{490\ nm}$ was measured after incubation. The extracellular protein was determined by the Coomassie Brilliant Blue method using bovine serum albumin as the standard. Twenty microlitres of the EPS sample were transferred into the wells of the 96-well plate. Two hundred microlitres of G-250 dye were added and mixed with the sample. The plate was incubated at 20 °C for 5 min and finally the $OD_{595\ nm}$ was measured to calculate the extracellular protein concentration. The extracellular DNA was extracted from the EPS sample using a TIANamp bacteria DNA kit (TIANGEN, China) and measured using a micro-ultraviolet spectrophotometer (NANODROP 2000; Thermo Fisher Scientific, USA).

Effect of benzalkonium chloride on gene expression. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to determine the relative expression levels of the target genes using the LightCycler 96 real-time PCR system (Roche, Switzerland) as described previously (Jiang et al. 2022). Primers used in this study have been reported previously (Jiang et al. 2021). To investigate the effect of BC on gene expression, *L. monocytogenes* 10403S was grown in BHI in the absence/presence of BC (4 μg·mL^{-1}). 16S rRNA was used as a reference gene.

Statistical analysis. Statistical analysis was performed using the SPSS (version 20.0). Differences were defined as significant at $P < 0.05$.

RESULTS AND DISCUSSION

Effect of benzalkonium chloride on *Listeria monocytogenes* 10403S growth. Table 1 shows the BC *MIC*s of five *L. monocytogenes* strains tested in this study. The highest BC *MIC* was observed in HL91 with 12 μg·mL^{-1} and the lowest was in HL38 with 4 μg·mL^{-1}. The *MIC* of BC against *L. monocytogenes* 10403S was 8 μg·mL^{-1}. Results from the growth curves of *L. monocytogenes* 10403S are presented in Figure 1 and Table 2. The presence of BC at sub-*MIC*s resulted in only

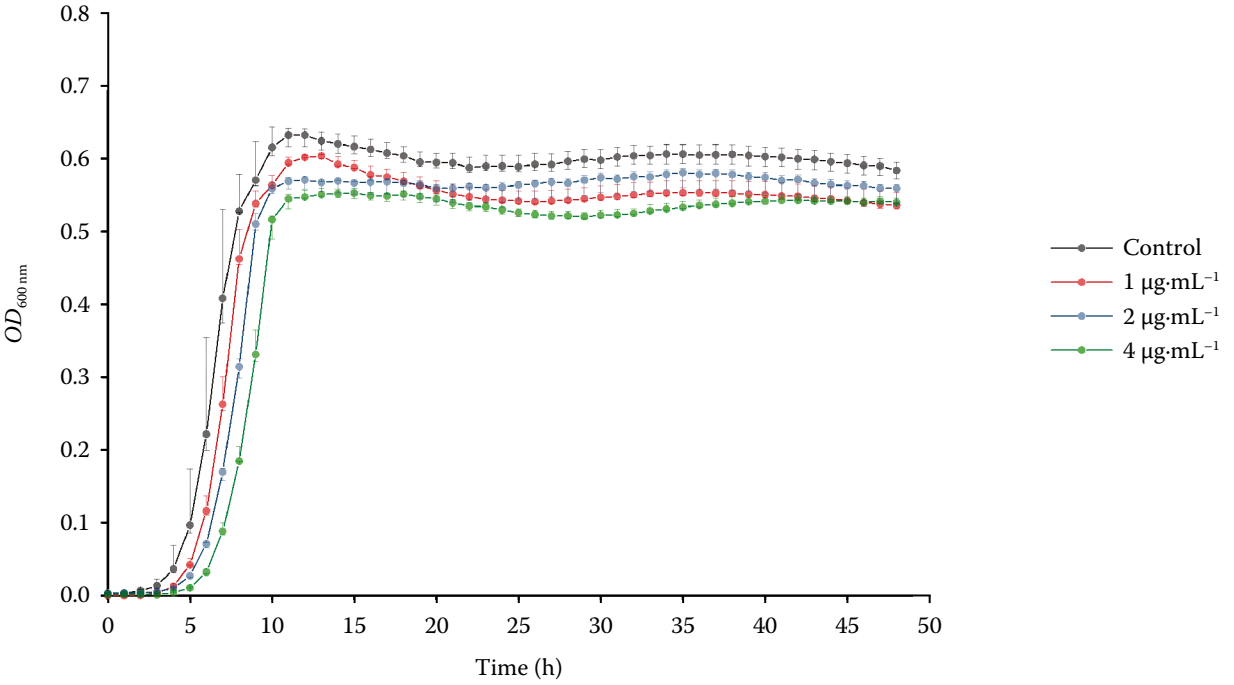

Figure 1. Growth curves of *Listeria monocytogenes* 10403S in the presence of benzalkonium chloride (BC) at sub-minimum inhibitory concentrations (sub-*MIC*s)

$OD_{600\ nm}$ – optical density

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Table 2. Growth parameters of *Listeria monocytogenes* 10403S in the presence of benzalkonium chloride (BC) at sub-minimum inhibitory concentrations (sub-*MIC*s)

BC concentration ($\mu g \cdot mL^{-1}$)	Growth parameter		
	lag-phase duration (h)	mean maximum growth rate ± SD ($OD_{600\ nm}$ units$\cdot h^{-1}$)	mean maximum optical density ± SD ($OD_{600\ nm}$ units)
0	5.334 ± 0.113	0.179 ± 0.005	0.626 ± 0.009
1	5.471 ± 0.132	0.179 ± 0.003	0.604 ± 0.002
2	6.054 ± 0.345	0.167 ± 0.013	0.576 ± 0.024
4	6.943 ± 0.850	0.159 ± 0.010	0.555 ± 0.033

SD – standard deviation; $OD_{600\ nm}$ – optical density

slightly longer lag-phase durations ($P > 0.05$), higher maximum growth rates ($P > 0.05$) and maximum optical densities ($P > 0.05$) compared to the control without BC. When exposed to lethal concentrations of BC, the survival rate at each time point was significantly lower ($P < 0.05$) than that of the control (Figure 2). Especially, no viable bacteria were detected after being exposed to 32 $\mu g \cdot mL^{-1}$ of BC (4 × *MIC*) for 20 min (Figure 2).

Many previous studies have reported BC susceptibility of *L. monocytogenes* (Ortiz et al. 2014; Bonneville et al. 2020; Rodríguez-Melcón et al. 2023). However, even the *MIC*s obtained from the same strain were not the same due to different susceptibility testing medium used in different studies. Our results showed that the *MIC*s of BC for all tested strains ranged from 4 to 12 $\mu g \cdot mL^{-1}$. The BC *MIC* of *L. monocytogenes* 10403S was 8 $\mu g \cdot mL^{-1}$. Sub-*MIC*s of BC at 1 (1/8 *MIC*), 2 (1/4 *MIC*), and 4 $\mu g \cdot mL^{-1}$ (1/2 *MIC*) were applied for growth curves and the results demonstrated that BC at sub-*MIC*s had no effect on growth. Thus, we decided to choose these concentrations of BC to investigate the effects of BC on biofilm formation. BC at lethal concentrations exhibited excellent bactericidal activity against *L. monocytogenes* 10403S in a concentration-dependent manner.

Effect of benzalkonium chloride on biofilm formation by *Listeria monocytogenes*. The presence

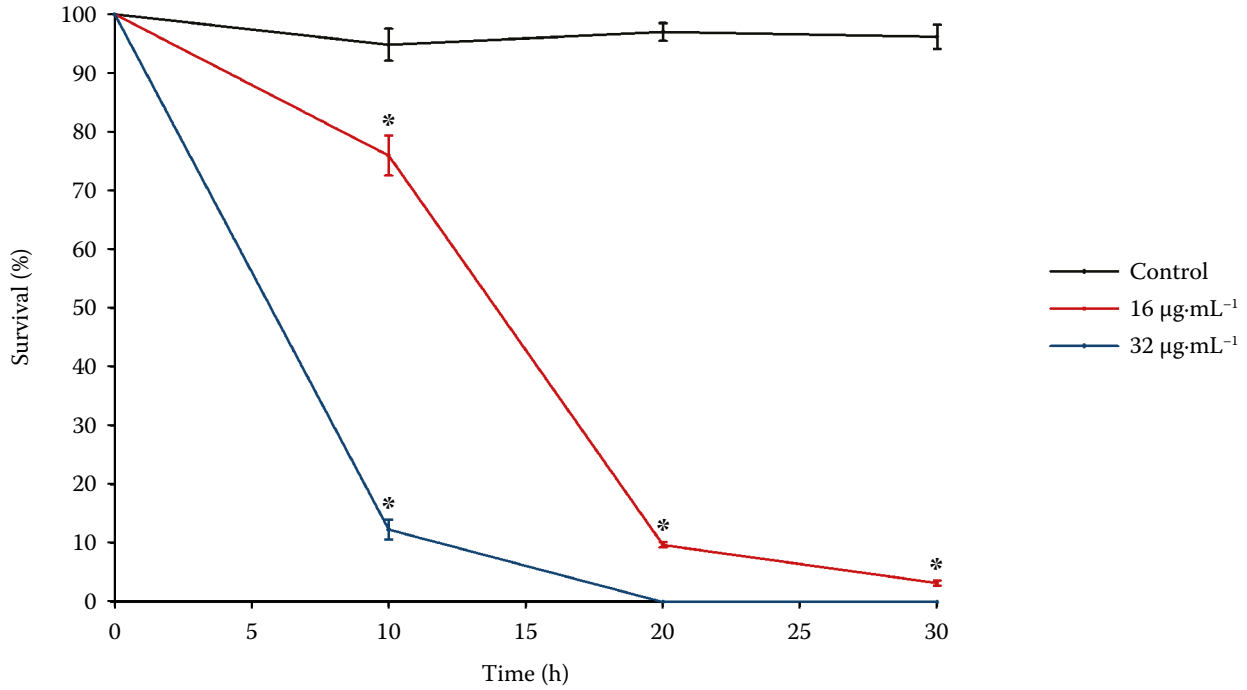

Figure 2. Killing curves of *Listeria monocytogenes* 10403S exposed to lethal concentrations of benzalkonium chloride (BC)

*Value statistically different from that of the control ($P < 0.05$)

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of BC at 1, 2, and 4 μg·mL^{-1} significantly reduced ($P < 0.05$) the biofilm biomass of 10403S by 18.3, 25.8, and 34.0%, respectively (Figure 3A). This suggests that sub-*MIC*s of BC could inhibit the biofilm formation of *L. monocytogenes* 10403S in a concentration-dependent manner. The addition of BC did not affect the viability of planktonic cells compared to the control (Figure 3B), indicating that the difference in biofilm forming ability was not due to a difference in growth.

The morphology of *L. monocytogenes* 10403S biofilms was observed using an inverted microscope (Figure 3C). The untreated *L. monocytogenes* biofilm was complete and dense. Following the BC treatment, biofilms showed a decrease in biofilm biomass. With the increase of BC concentration, increasing dispersal and decreased adherence of biofilms were also observed.

Effects of BC on the biofilm formation of the other *L. monocytogenes* strains were also investigated. As shown in Figure 3D, the biofilm biomass of EGD-e, HL12, HL38, and HL91 was reduced by 28.8, 23.6, 38.2, and 16.7%, respectively, in the presence of BC (2 μg·mL^{-1}).

Ortiz et al. (2014) reported that sub-*MIC*s of BC can inhibit the biofilm formation of both BC-resistant

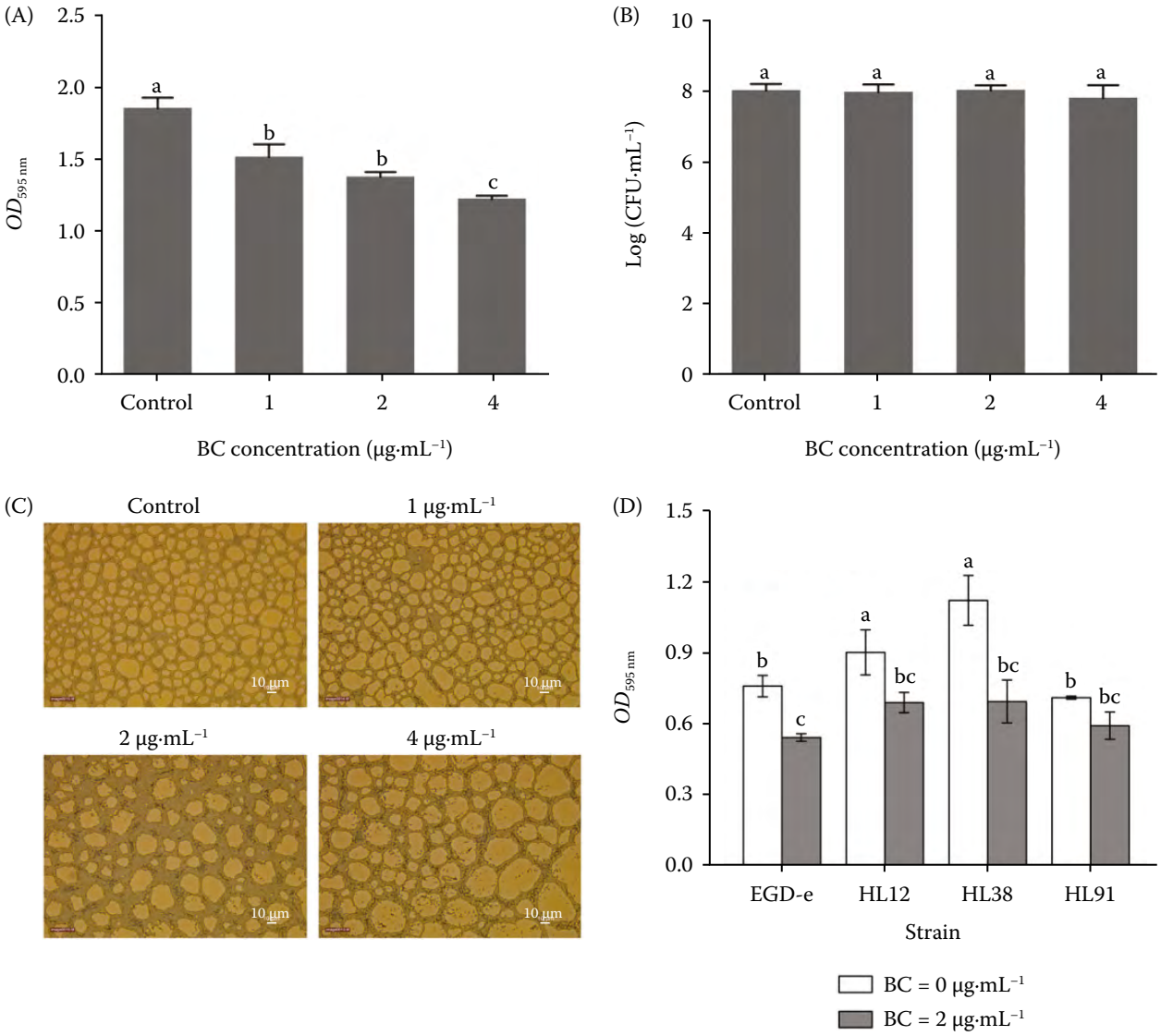

Figure 3. Effect of benzalkonium chloride (BC) on the biofilm formation of *Listeria monocytogenes* 10403S: (A) Biofilm biomass, (B) the number of planktonic cells in the culture supernatant, (C) microscope observation, and (D) biofilm biomass of four strains

a–c – significant differences at $P < 0.05$; error bars – standard deviation; the control refers to biofilms incubated in brain heart infusion (BHI) without BC; $OD_{595\ nm}$ – optical density; CFU – colony forming unit

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strains (*MIC* ≥ 10 μg·mL^{-1}) and BC-sensitive strains (*MIC* ≤ 2.5 μg·mL^{-1}) of *L. monocytogenes*. This suggests that the inhibitory effect of sub-*MIC*s of BC on biofilm production by *L. monocytogenes* could be not related to BC susceptibility of the strains.

Effect of benzalkonium chloride on produced biofilms by *Listeria monocytogenes*. BC at lethal concentrations of 8, 16, and 32 μg·mL^{-1} significantly reduced the mature biofilm by 26.2, 34.3, and 42.7%, respectively (Figure 4A). Also surviving cells in mature biofilms were measured by colony counting. BC at 8, 16, and 32 μg·mL^{-1} resulted in a significant decrease ($P < 0.05$) in the cell viability of biofilms compared to the control (Figure 4B). Images from the inverted microscope showed decreased adherence in BC-treated biofilms (Figure 4C). When exposed to 16 μg·mL^{-1} of BC, the mature biofilms of EGD-e, HL12, HL38, and HL91 were decreased by 39.8, 38.4, 55.8, and 21.0%.

Mature biofilms are significantly more resistant to disinfectants and difficult to remove (Jiang et al. 2022). Strantzali et al. (2021) reported that a less than 2-log reduction in the number of living cells in mature biofilms produced by *Salmonella* Typhimurium DT193 was observed when BC was applied at 70 μg·mL^{-1} (3.5 × *MIC*). In this study, the reduction in the viable cell number in *L. monocytogenes*

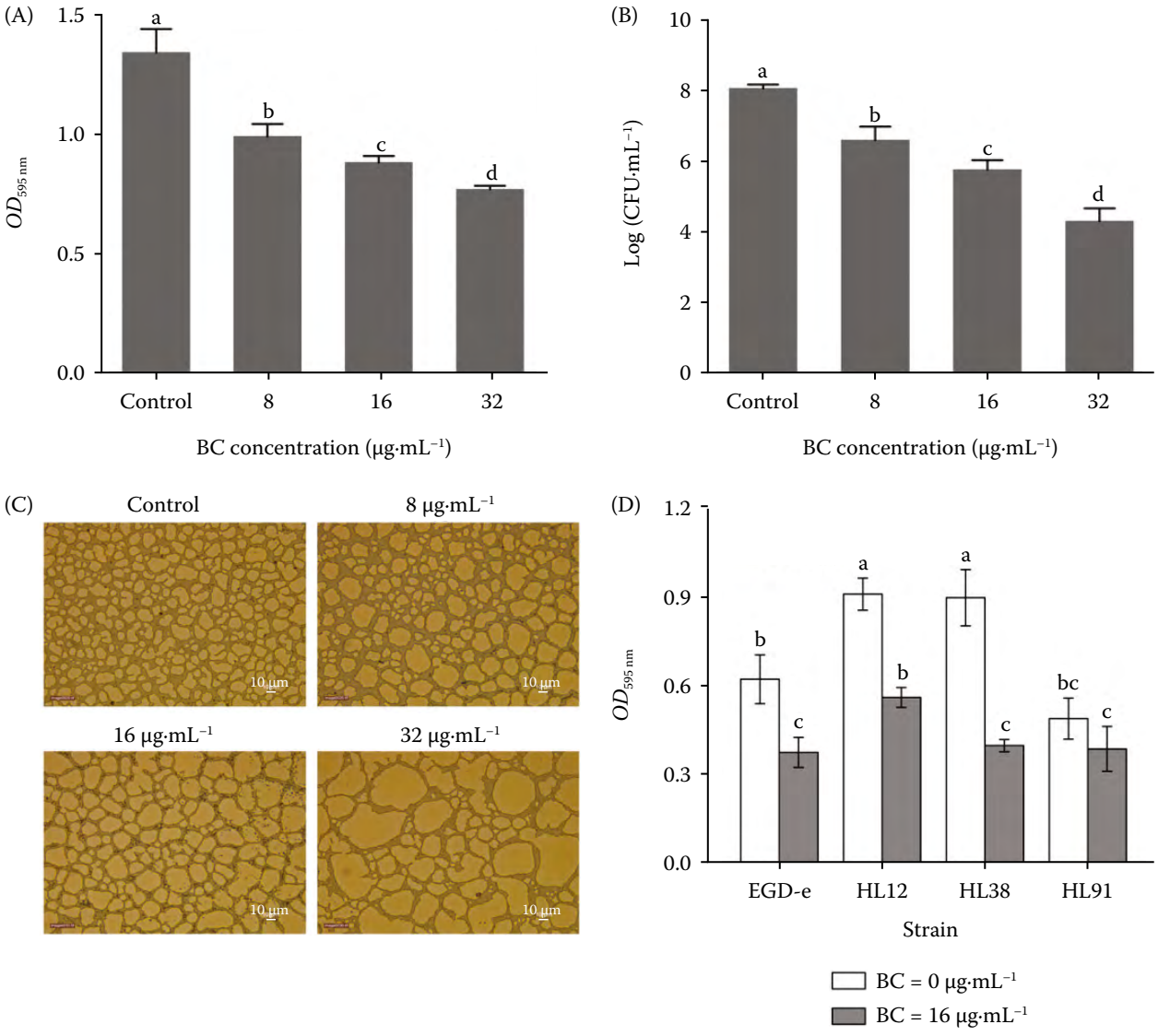

Figure 4. Effect of benzalkonium chloride (BC) on produced biofilms of *Listeria monocytogenes* 10403S: (A) Biofilm biomass, (B) surviving cells in biofilms, (C) microscope observation, and (D) biofilm biomass of four strains

a–d – significant differences at $P < 0.05$; error bars – standard deviation; the control refers to mature biofilms without BC treatment; $OD_{595\ nm}$ – optical density; CFU – colony forming unit

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10403S mature biofilms exceeded 2 logs when treated with 2 × *MIC* of BC (16 μg·mL^{-1}). It suggests that the eradication effect of BC on *L. monocytogenes* biofilms is superior to that on *Salmonella* biofilms.

Effect of benzalkonium chloride on biofilm extracellular polymeric substances. After incubation in BHI broth with sub-*MIC*s of BC, biofilm EPS were measured in this study (Figure 5). When treated with BC at 1, 2, and 4 μg·mL^{-1}, the extracellular polysaccharide was significantly decreased ($P < 0.05$) by 27.5, 31.4, and 40.5%, respectively, and the extracellular protein was significantly reduced ($P < 0.05$) by 33.8, 38.3, and 50.2%, respectively. However, no obvious difference ($P > 0.05$) in the amount of extracellular DNA was observed between the BC treatment groups and the control.

EPS in the mature biofilms were also quantified after exposure to lethal concentrations of BC. When treated with BC at 8, 16, and 32 μg·mL^{-1}, the extracellular polysaccharide was significantly decreased ($P < 0.05$) by 29.5, 45.7, and 55.4%, respectively, and the extracellular protein was significantly reduced ($P < 0.05$) by 51.1, 66.9, and 87.8%, respectively. The content of extracellular DNA in the BC treatment groups was similar to that in the control.

EPS have been recognised as an important component of bacterial biofilms, which plays a structure-stabilising and protective role in biofilm (Aqawi et al. 2021). Biofilm EPS are mainly composed of exopolysaccharides, extracellular proteins and extracellular DNA (Gautam et al. 2022; Catal et al. 2024). In *L. monocytogenes* biofilms, extracellular proteins account for the highest proportion of EPS (Nwaiwu et al. 2021). Previous studies have reported that extracellular proteins and extracellular DNA play important roles in the initial adhesion and in the early stage

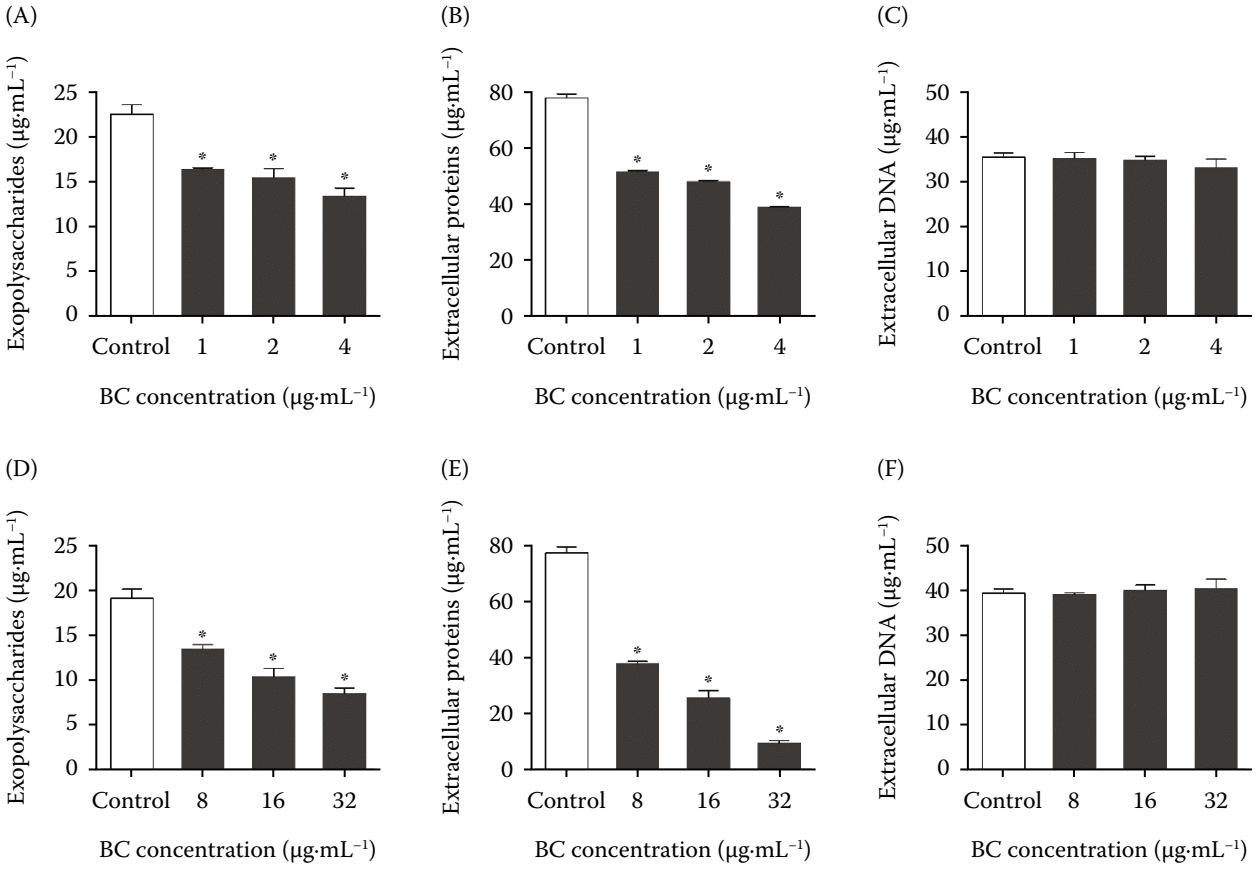

Figure 5. Effect of benzalkonium chloride (BC) on the content of extracellular polymeric substances (EPS) in the biofilm matrix of *Listeria monocytogenes* 10403S. Biofilms were incubated in the presence of BC (1, 2, and 4 μg·mL^{-1}, respectively) for 48 h, and then the amount of (A) exopolysaccharides, (B) extracellular proteins, and (C) extracellular DNA in biofilms was determined. After exposure to BC (8, 16, and 32 μg·mL^{-1}, respectively) for 30 min, the content of (D) exopolysaccharides, (E) extracellular proteins, and (F) extracellular DNA in mature biofilms was quantified.

*Value statistically different from that of the control ($P < 0.05$)

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of biofilm formation in *L. monocytogenes* (Nwaiwu et al. 2021; Banerji et al. 2022). When incubated in the presence of sub-*MIC*s of BC, both exopolysaccharides and extracellular proteins in biofilms were reduced, while the content of extracellular DNA was not affected by BC. These results indicate that sub-*MIC*s of BC can inhibit the biofilm formation of *L. monocytogenes* by reducing the secretion of exopolysaccharides and extracellular proteins. A similar phenomenon was observed in mature biofilms when exposed to lethal concentrations of BC. Specifically, BC had a greater impact on extracellular proteins than on exopolysaccharides in mature biofilms. The results suggest that BC at lethal concentrations can destroy the biofilm structure by targeting extracellular proteins and exopolysaccharides in *L. monocytogenes*.

Effect of benzalkonium chloride on gene expression. The relative gene expression of quorum sensing (QS) genes (*luxS* and *agrBDCA*), flagellar synthesis gene (*flaA*) and motility genes (*motA* and *motB*) was detected in the presence of BC (Figure 6). Results showed that *luxS* and the *agr* operon were significantly upregulated ($P < 0.05$) when exposed to BC at 4 μg·mL^{-1}. However, the expression levels of *flaA*, *motA*, and *motB* were significantly inhibited ($P < 0.05$) by BC.

The QS system is an important regulatory mechanism in bacteria that controls a variety of physiological activities, such as biofilm formation (Hammer and Bassler 2003; Waters and Bassler 2005). Two QS systems, LuxS and Agr, have been identified in *L. monocytogenes*. It was reported that LuxS is involved in the repression of biofilm formation in *L. monocytogenes* (Belval et al. 2006). An increased expression level of *luxS* was observed in the presence of BC at 1/2 *MIC*. This may be one of the reasons that sub-*MIC*s of BC can inhibit the biofilm formation in *L. monocytogenes*. The Agr QS system consists of the four-gene operon *agrBDCA* and its positive regulation of the biofilm formation has been reported in previous studies (Gandra et al. 2019; Lee and Wang 2020). Our results showed that BC could induce the expression levels of the *agr* genes. It seems that the increased expression of the *agr* operon and reduced biofilms in the presence of BC are contradictory. Actually, the biofilm formation of *L. monocytogenes* is also regulated by other mechanisms than

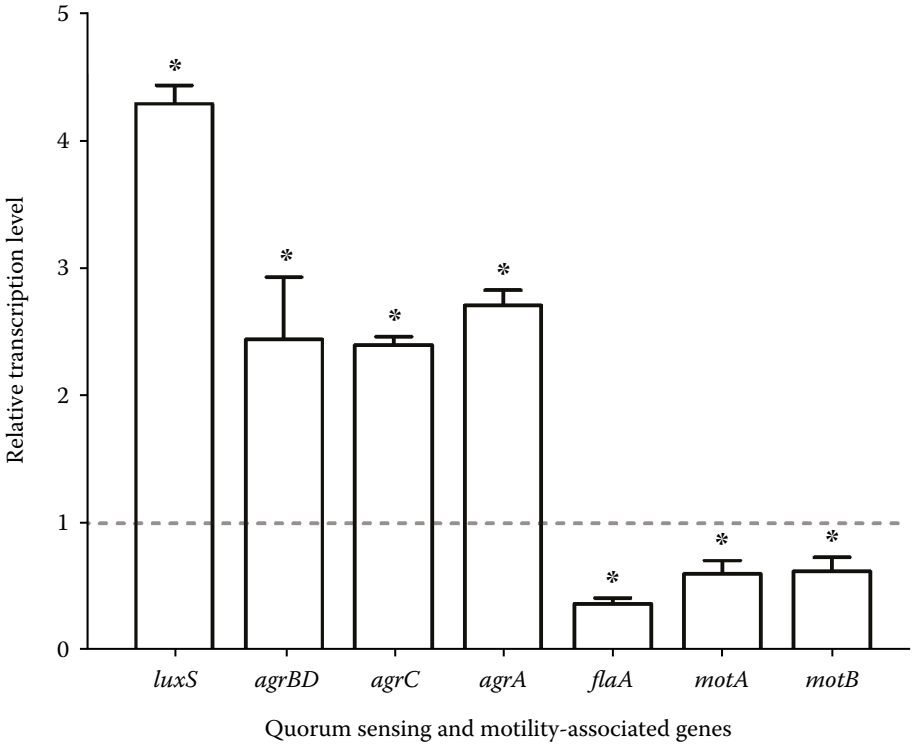

Figure 6. Relative expression levels of quorum sensing (QS) genes and motility-associated genes in *Listeria monocytogenes* 10403S in brain heart infusion (BHI) broth with or without benzalkonium chloride (BC). Results are presented as fold changes relative to the expression level of the target gene *L. monocytogenes* 10403S grown in BHI.

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the Agr QS system. Thus, it was reasonable that the inconsistent effects of BC on the Agr QS system and biofilm formation in *L. monocytogenes* were observed in the present study.

Flagellum-mediated motilities play a significant role in the first stage of biofilm formation (Lau et al. 2023). In our study, effects of BC on flagellar motility genes were also investigated. Results showed that the expression of these genes was inhibited by BC at 1/2 *MIC*, which could be the possible reason for reduced ability to form biofilms by *L. monocytogenes* in the presence of sub-*MIC* of BC.

CONCLUSION

In summary, the BC disinfectant can inhibit the biofilm formation and remove mature biofilms of *L. monocytogenes* strains, suggesting the anti-biofilm activity of BC against *L. monocytogenes*. Additionally, upregulation of the QS gene *luxS* and downregulation of flagellum motility genes *flaA*, *motA*, and *motB* was observed in the presence of BC, which may result in biofilm formation inhibited by BC.

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Received: March 7, 2024
Accepted: June 21, 2024
Published online: August 7, 2024