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Susceptibility of *Listeria monocytogenes* planktonic cultures and biofilms to sodium hypochlorite and benzalkonium chloride

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ABSTRACT

The susceptibility of four *L. monocytogenes* isolates from pork to sodium hypochlorite (SHY) and benzalkonium chloride (BZK) was tested. Minimum inhibitory concentration (MIC) values of 3500 ppm (SHY), or between 3 ppm and 13 ppm (BZK), were found. Minimum bactericidal concentration (MBC) values ranged from 3500 ppm to 4500 ppm (SHY), and from 3 ppm to 14 ppm (BZK). The effect of SHY and BZK on the architecture and cellular viability of 24-h-old biofilms formed by such strains on polystyrene was determined through confocal laser scanning microscopy (CLSM) in conjunction with fluorescent dyes for live cells (SYTO 9) and dead cells (propidium iodide). Strains were able to form biofilm (biovolume values in the observation field of 14,161 μm^2 ranged between 103,928.3 ± 6730.2 μm^3 and 276,030.9 ± 42,291.9 μm^3). Treatment of biofilms for 10 min with SHY (1MIC or 1.5MIC) or BZK (0.5MIC, 1MIC or 1.5MIC) decreased the biovolume of live (potentially dangerous) cells. SHY reduced the cellular viability of biofilms by more than 90%. On the other hand, BZK was able to remove most biofilm mass (live and dead cells), but decreased cellular viability only to a lesser extent, this suggesting strong biofilm detachment and dissemination of live cells.

1. Introduction

Listeria monocytogenes is a Gram-positive, rod-shaped, facultative anaerobic, non-spore-forming bacterium which is responsible for listeriosis, a rare but deadly disease, whose main transmission route to humans is believed to be through the consumption of contaminated food (Ryser and Marth, 2007). In 2017, 2480 confirmed cases of invasive human listeriosis were reported in the European Union (0.48 cases per 100,000 individuals). The fatality rate was 13.8% (225 deaths among the 1633 confirmed cases with known outcome), the highest value of all among foodborne diseases (EFSA and ECDC, 2018). Approximately 1600 infections and 260 deaths due to listeriosis occur annually in the United States, with an incidence rate of 0.26 per 100,000 individuals in 2014 (Gurtler et al., 2017). The elderly, pregnant women, new-born children and immunocompromised adults are those most affected by this disease, although people without these risk factors can also be infected. Septicaemia, meningitis, encephalitis, miscarriage and stillbirth are common clinical presentations (Capita et al., 2001).

L. monocytogenes is a ubiquitous bacterium, which is able to grow in different adverse environmental conditions including temperature (0.4 °C to 4 °C), pH values (4.0–9.6), high salt content (10%–20%), and low oxygen levels, and constitutes a major concern for the food industry (Sadekuzzaman et al., 2017). *L. monocytogenes* is capable of adhering to, and forming biofilms on, most surfaces that are found in food processing facilities, such as polystyrene, glass or stainless steel (Di Bonaventura et al., 2008). Biofilms are structured microbial communities enclosed in a self-produced matrix of hydrated extracellular polymeric substances (EPS) and are adhered to an inert or living surface (González-Machado et al., 2018). The ability to form biofilms is a key factor in the persistence of *L. monocytogenes* in food environments for long periods, and these structures represent a potential source of contamination of food products, leading to critical problems in terms of public health and economic impact (Pilchová et al., 2014). In general, cells in biofilms show a higher resistance to environmental challenges, such as desiccation, UV light or sanitizers, than their planktonic counterparts in suspension (Capita et al., 2014). Consequently, control of biofilms remains difficult.

Chlorine-based disinfectants, such as sodium hypochlorite, are oxidizing compounds widely used in the food industry due to their broad-

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spectrum bactericidal activities, high efficacy and low cost (Waghmare and Annapure, 2015). Quaternary ammonium compounds (QAC), such as benzalkonium chloride, are cationic surfactants that act through the disruption of lipid membrane bilayers, being effective against several photogenic microorganisms, especially Gram-positive bacteria (Ortiz et al., 2014). As previously suggested (Capita et al., 2014), disinfectants are sometimes used at sub-minimum inhibitory concentrations (sub-MICS) in the food industry (e.g. through a failure to apply a sufficient dose of disinfectant, insufficient cleaning before disinfection, or the inappropriate storage of biocides). Understanding the response of *L. monocytogenes* to conditions that may occur in the processing environment would assist in the development of effective disinfection strategies. However, it would seem that reports quantitatively assessing the effect of sub-MICs of SHY and BZK on structural parameters (e.g. biovolume, surface coverage, thickness or roughness) and cellular viability of *L. monocytogenes* biofilms are lacking. Thus, the aim of this study was to use confocal laser scanning microscopy (CLSM) and image analysis to evaluate the effect of various concentrations (0.5MIC, 1MIC and 1.5 MIC) of two biocides commonly used in the food industry (sodium hypochlorite and benzalkonium chloride) on the structural parameters and cellular viability of the biofilms formed on polystyrene by four strains of *L. monocytogenes*. Additionally, the minimum inhibitory concentrations (MICs) and the minimum bactericidal concentrations (MBCs) of such biocides were determined for planktonic cultures.

2. Materials and methods

2.1. L. monocytogenes strains and culture conditions

Four *L. monocytogenes* isolates (serotype 1/2a) from pork were used (LM2, LM6, LM7 and LM12). The strains were kept in tryptone soya broth (TSB, Oxoid Ltd., Hampshire, England) supplemented with 20% (v/v) glycerol at −80 °C. Prior to experiments, frozen cells were sub-cultured twice in TSB at 37 °C. Working cultures were kept at 4 °C ± 1 °C on plates of tryptone soya agar (TSA, Oxoid) and were sub-cultured monthly.

2.2. Biocides

Two compounds were tested: sodium hypochlorite (10% active chlorine; SHY, Sigma-Aldrich, Steinheim, Germany) and benzalkonium chloride (BZK, Fluka, Deisenhofen, Germany). Solutions of chemicals were prepared aseptically in sterile distilled water immediately before experiments. Pure active biocides were used to gain a better estimate of the eventual resistance of biofilms, instead of testing commercial sanitizers that include multiple substances.

2.3. Determination of MICs and MBCs

Susceptibilities of planktonic cells and biofilms were tested in order to improve knowledge about *L. monocytogenes* behaviour (both planktonic and sessile cells) in the presence of food-grade disinfectants. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) values for planktonic *L. monocytogenes* cells were established using a micro-dilution broth method in accordance with the CLSI (2013) guidelines.

For the experiment, 100-well polystyrene microplates (Oy Growth Curves Ab Ltd., Helsinki, Finland) were used. Five colonies of each strain were taken from TSA plates, inoculated into 10 ml of TSB (Oxoid) and incubated at 37 °C. Previous experiments had shown that after 24 h of incubation such bacterial cultures contain approximately 5×10^8 cfu/ml. Three decimal dilutions were performed in TSB. Wells were filled with 20 μl of the chemical solution (a range of concentrations was used for each biocide, from 250 ppm to 5000 ppm, at intervals of 250 ppm, for SHY, and from 1 ppm to 20 ppm, at intervals of 1 ppm, for BZK) and 180 μl of the third dilution of this bacterial culture in order to give a final concentration in the well of approximately 5×10^5 cfu/ml. The inoculum concentration was confirmed by plating. The microwell plates were incubated at 37 °C in the automated turbidimetric-based system Bioscreen C MBR (Oy Growth Curves Ab Ltd.). Positive controls (200 μl of inoculum at 5×10^5 cfu/ml) were included in each experiment, as were negative (180 μl of TSB and 20 μl of chemical solution) controls. The experiments were replicated five times on separate days. The MIC was established as the lowest biocide concentration necessary to prevent growth after 48 h of incubation (Capita et al., 2014), being growth of the strain determined by measuring the optical densities (OD) at 580 nm.

For minimum bactericidal concentration (MBC) calculations, 100 μl of the wells without observed growth were surface-plated on TSA and incubated at 37 °C for 72 h. The MBC was established as the lowest biocide concentration necessary to prevent growth on TSA, i.e. to reduce at least 10,000 times the concentration of *L. monocytogenes* in the well (from 10^5 cfu/ml to lower than 10 cfu/ml). For MIC and MBC calculation, five independent experiments were performed for each strain and chemical compound on different days.

2.4. Biofilm formation

An analysis of the structure of the biofilm was undertaken using the method previously described by Capita et al. (2014), with some modifications. Briefly, cultures were grown at 37 °C for 18 h, and appropriate (two-fold) dilutions in TSB were made to obtain a concentration of approximately 10^6 cfu/mL. A volume of 250 μl was added to the wells of Nunc™ MicroWell™ 96-Well Optical-Bottom Plates with Polymer Base (Thermo Fisher Scientific, New Hampshire), having high optical quality, low fluorescent background and overall flatness, which allowed high resolution imaging. After one hour of adhesion at 37 °C, the wells were rinsed with 150 mM of NaCl in order to eliminate any non-adherent bacteria, before being refilled with 250 μl of TSB and incubated for 24 h at 37 °C.

2.5. Treatment of biofilms

The wells were rinsed with 150 mM of NaCl and refilled with TSB (control) or with TSB containing SHY or BZK at different concentrations (0.5MIC, 1MIC and 1.5MIC were used). After 10 min of exposure at room temperature, the wells were rinsed with 150 mM of NaCl.

2.6. Image analyses

To differentiate between live and dead bacteria, two fluorescent dyes, SYTO9 and propidium iodide (PI), were used. The red fluorescent PI penetrates only those cells which have disrupted membranes and is generally excluded from viable cells. In contrast, the green-fluorescent nucleic acid stain SYTO9 enters both live and dead bacterial cells. The fluorescent signal of SYTO9 is strongly enhanced when it is bound to nucleic acid and it shows a low intrinsic fluorescence signal when unbound. When both dyes are present, PI exhibits a stronger affinity for nucleic acids than SYTO9, and hence, SYTO9 is displaced by PI. Thus, viable cells having intact membrane emit a green fluorescence, whereas dead bacteria having a damaged membrane emit a red fluorescence (Buzón-Durán et al., 2017).

A volume of 2 μl of a 1:1 mixture of SYTO9 (stock 3.34 mM in DMSO) and propidium iodide (PI; stock 20 mM in DMSO) fluorescent dyes from the BacLight Viability Kit (Invitrogen, Carlsbad, California) was added to 1000 μl of TSB, and 250 μl of this solution was added to each well. The plate was then incubated in the dark at 37 °C for 20 min to enable fluorescent labelling of the bacteria.

Confocal laser Scanning Microscopy (CLSM) image acquisition was performed using a Nikon Eclipse TE 2000-U confocal laser scanning microscope with EZ-C13.60 software (Nikon Instruments Inc., New York). The biofilms were observed with a forty magnification (40×)

lens. Fluorescence was detected by excitation at 488 nm (argon laser), and emissions were collected with a 590/50 (SYTO9) or a 650LP (PI) bandpass filter.

Three stacks of horizontal plane images (512 × 512 pixels corresponding to 119 × 119 μm) with a z-step of 1 μm were acquired for each biofilm from three different areas in the well. Three independent experiments were performed for each strain and chemical treatment on different days. Thus, a total of 252 CLSI images were obtained: 4 strains x 7 treatments (2 chemicals x 3 concentrations + controls) x 3 replicates x 3 experiments. For image analysis, three-dimensional projections of the structure of the biofilms were reconstructed using the Easy3D function of the IMARIS 9.0 software package (Bitplane, Zurich, Switzerland).

The quantitative structural parameters of the biofilms were calculated using the BioRCA 1.4 software, developed by some members of our Research Group using the *Lazarus* Integrated Development Environment (IDE) (Buzón-Durán et al., 2017; Capita et al., 2017). From a threshold value, each pixel is labelled as empty, green (live) or red (dead). With this information, a three-dimensional model of the biofilm under study is generated and the structural parameters are calculated. This computer program allowed quantification of the total biofilm population (comprising both live and dead bacteria) and of the independent subpopulations represented by green fluorescence emitted by SYTO 9 (from cells with intact membranes) and red fluorescence emitted by PI (from bacteria with damaged membranes). The biovolume represented the overall volume of cells (μm^3) in the observation field (14,161 μm^2) and provided an estimate of the biomass in the biofilm. Surface coverage (%) reflected the efficiency of substratum colonization by the populations of bacteria. Roughness provided a measure of how much the thickness of the biofilm varied and was thus an indicator of biofilm heterogeneity (Murga et al., 1995). A roughness with a value of zero indicates a biofilm of uniform thickness, and a value close to 1 describes a patchy biofilm. The maximum thickness (μm) of biofilms was determined directly from the confocal stack images.

2.7. Statistical analysis

The quantitative structural parameters of biofilms were compared for statistical significance using one-way analysis of variance techniques. Mean separations were obtained using Duncan's multiple range test, and significant differences were established at the 5% ($P < 0.05$) level. Data were processed using the Statistica® 8.0 software package (Statsoft Ltd., Tulsa, Oklahoma).

3. Results

3.1. Susceptibility of planktonic cultures to SHY and BZK

Table 1 shows the MIC and MBC values of SHY and BZK for *L. monocytogenes*. The strains' response to SHY, expressed in MIC or MBC values, was less variable than to BZK. MIC_{SHY} values of 3500 ppm were observed for all isolates. Data for SHY were not correlated with those obtained for BZK. BZK displayed a range of antimicrobial efficacy, with MICs varying from 3 ppm (LM2) to 13 ppm (LM6). MBC_{SHY} values ranged from 3500 ppm (LM6 and LM7) to 4500 ppm (LM2 and LM12), and MBC_{BZK} values between 3 ppm (LM2) and 14 ppm (LM6). MBC values were greater than or equal to MIC values for all strains.

3.2. Ability of L. monocytogenes to form biofilm

Biofilms were tested on polystyrene-bottomed microtitre plates to facilitate confocal imaging. Representative twenty-four-hour-biofilm structures observed using CLSM for the four strains under study are shown in Fig. 1. All isolates were able to produce rough biofilms of moderate biovolume that covered most of the glass surface available after 24 h of incubation.

Table 1
Minimum inhibitory concentration (ppm) and minimum bactericidal concentration (ppm) values for sodium hypochlorite and benzalkonium chloride in respect of four strains of *L. monocytogenes*.

Parameter	Strain			
	LM2	LM6	LM7	LM12
MIC_{SHY}	3500	3500	3500	3500
MBC_{SHY}	4500	3500	3500	4500
MIC_{BZK}	3	13	10	9
MBC_{BZK}	3	14	12	9

Five replications were performed for each condition; MIC_{SHY}, minimum inhibitory concentration of sodium hypochlorite; MBC_{SHY}, minimum bactericidal concentration of sodium hypochlorite; MIC_{BZK}, minimum inhibitory concentration of benzalkonium chloride; MBC_{BZK}, minimum bactericidal concentration of benzalkonium chloride.

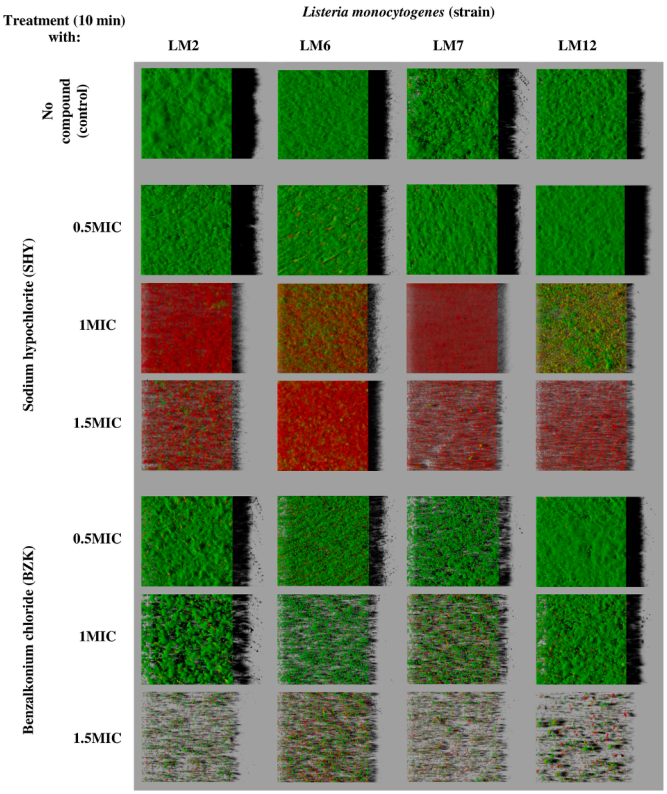

Fig. 1. Three-dimensional projections of biofilm structures of four *L. monocytogenes* isolates after treatment for 10 min with sodium hypochlorite (SHY) or benzalkonium chloride (BZK) at different concentrations. Images (119 μm × 119 μm) were reconstructed from confocal z-stacks using IMARIS software, with the shadow projections on the right.

Structural parameters (biovolume, substratum coverage, maximum thickness and roughness) were obtained from confocal stack images in order to quantify biofilm structures with numerical data that would allow statistical analysis. The results obtained revealed a marked variability in the structure of biofilms between the four strains. Numerical data for structural parameters of non-treated biofilms ranged from 103,928.3 ± 6730.2 μm^3 (LM12) to 276,030.9 ± 42,291.9 μm^3 (LM2) in the observation field (14,161 μm^2) for biovolume, from 93.95 ± 6.48% (LM7) to 99.99 ± 0.01% (LM2 and LM6) for surface coverage, from 16.10 ± 0.24 μm (LM12) to 31.23 ± 2.07 μm (LM2) for maximum thickness, and from 0.1039 ± 0.0009 (LM6) to 0.3267 ± 0.1453 (LM7) for roughness, respectively (Tables 2–5). Even though all strains efficiently colonized polystyrene, showing minimal

Table 2
Biovolume (μm^3) values in the observation field (14.161 μm^2) obtained for the biofilms by four *L. monocytogenes* strains after exposure for 10 min to different concentrations of sodium hypochlorite or benzalkonium chloride.

Treatment (10 min) with:		*Listeria monocytogenes* (strain)			
		LM2	LM6	LM7	LM12
No compound (control)		276,030.9 ± 42,291.9$^{a}_{a}$	224,684.4 ± 5190.3$^{b}_{a}$	129,028.1 ± 58,348.9$^{c}_{a}$	103,928.3 ± 6730.2$^{c}_{a}$
Sodium hypochlorite (SHY)	0.5MIC	145,730.2 ± 26,417.0$^{a}_{b}$	153,628.0 ± 4225.1$^{a}_{b}$	147,023.4 ± 31,614.0$^{a}_{a}$	219,509.8 ± 16,390.8$^{b}_{b}$
	1MIC	89,065.8 ± 40,853.6$^{a}_{bc}$	108,928.9 ± 35,742.9$^{a}_{cd}$	21,033.7 ± 10,098.9$^{b}_{b}$	54,484.6 ± 20,416.3$^{ab}_{c}$
	1.5MIC	27,402.2 ± 2738.7$^{a}_{c}$	122,920.3 ± 12,910.1$^{b}_{c}$	19,223.3 ± 589.1$^{a}_{b}$	26,459.5 ± 2595.5$^{a}_{cd}$
Benzalkonium chloride (BZK)	0.5MIC	50,763.4 ± 44,097.4$^{a}_{c}$	86,593.4 ± 16,910.5$^{a}_{d}$	52,501.0 ± 46,237.4$^{a}_{b}$	74,377.8 ± 55,580.6$^{a}_{ac}$
	1MIC	66,737.8 ± 32,950.7$^{a}_{c}$	26,241.1 ± 5639.9$^{b}_{e}$	22,952.5 ± 11,663.4$^{b}_{b}$	69,368.7 ± 19,632.0$^{a}_{ac}$
	1.5MIC	34,227.8 ± 34,442.5$^{a}_{c}$	14,538.2 ± 6411.5$^{a}_{e}$	20,745.8 ± 24,752.4$^{a}_{b}$	10,495.6 ± 1028.1$^{a}_{d}$

Data (mean ± STD) are the average of nine determinations. Means in the same row with no superscript letters in common are significantly different ($P < 0.05$). Means in the same column with no subscript letters in common are significantly different ($P < 0.05$).

differences in surface coverage, biofilms formed by strains LM2 and LM6 showed the highest biovolume, mainly because their higher thickness.

3.3. Effect of SHY and BZK on the structural parameters and viability of biofilms formed by L. monocytogenes

In the present work, the effect of two disinfectants commonly used in food processing facilities (sodium hypochlorite and benzalkonium chloride) against biofilms formed by four *L. monocytogenes* strains of food origin was analysed. Biofilms were exposed for 10 min to different concentrations (0.5MIC, 1MIC or 1.5MIC) of SHY or BZK. The architecture and viability of biofilms treated with the two disinfectants were evaluated by CLSM. Representative CLSM images of biofilms are shown in Fig. 1, and structural parameters are given in Tables 2–5

Antimicrobials applied at 0.5MIC reduced the biovolume of biofilms ($P < 0.05$) in the case of LM2 and LM6 (SHY), and LM2, LM6 and LM7 (BZK). On the other hand, disinfectants at 1MIC or 1.5MIC (especially BZK at 1.5MIC) substantially reduced the biovolume, surface coverage and maximum thickness of biofilms, while increasing their roughness.

Two types of cells were observed: green cells represented intact or viable bacteria, whereas red cells represented dead bacteria with damaged membranes. Green structures were observed in the control (non-exposed) biofilms (Fig. 1). A matrix of green cells with a few red cells was observed in micro-wells that were exposed to 0.5MIC SHY. In contrast, a greater number of red cells was observed in samples treated with SHY at 1MIC or 1.5MIC. BZK only slightly reduced cellular viability in the biofilm. Fig. 2 shows the percentages of live and dead cells for each strain and chemical treatment. The survival rates of cells in biofilms varied widely among the strains and with the type of treatment applied. The percentage of dead (red-stained) cells in controls (non-treated biofilms) ranged from 0.21 ± 0.12% (LM2) to 1.77 ± 0.20% (LM7). The cellular viability of the biofilm was barely affected by exposure to SHY or BZK at a low concentration (0.5MIC). Treatment with SHY at 1MIC or 1.5MIC resulted in significant and wide-ranging cell death (from 35.84 ± 24.00% for LM12 to 99.00 ± 0.95% for LM7). The viability of biofilms also decreased after exposure to BZK at 1.5MIC (data for dead cells ranged between 38.12 ± 1.42% for LM2 and 77.32 ± 22.34% for LM7). Treatment with BZK at 1MIC modified the percentage of red cells with regard to control (untreated) biofilms, but only for strains LM2 and LM7.

Because only live cells are potentially dangerous, the biovolume of live (green) cells in control and treated biofilms was quantified by CLSM (Fig. 3). SHY at 0.5MIC did not reduce the biovolume of live cells relative to untreated biofilms in biofilms formed by LM7 and LM12. In contrast, the remaining treatments (SHY at 1MIC or 1.5MIC and BZK at 0.5MIC, 1MIC and 1.5MIC) decreased ($P < 0.05$) the biovolume of live cells in biofilms formed by all strains.

4. Discussion

4.1. Susceptibility of planktonic cultures to SHY and BZK

Several authors have assessed the resistance to biocides of *L. monocytogenes* strains. However, it would appear that this is the first piece of work simultaneously determining the effect of two food-grade biocides (SHY and BZK) on planktonic cultures and structural parameters of the biofilms by several *L. monocytogenes* isolates from food.

MIC values observed for SHY were similar than data found by other researchers testing *L. monocytogenes* isolates from foodstuffs or culture collections (512 ppm by Gao and Liu, 2014; 2500 ppm by Lundén et al., 2003). The values observed in this study for MICs of BZK (3–13 ppm) are also in agreement with the findings of other authors testing *L. monocytogenes* from different origins: from 4 ppm to 7 ppm (Aase et al., 2000), from 0.63 ppm to 5 ppm (Lundén et al., 2003), from 1.87 ppm to 15 ppm (Soumet et al., 2005) or from 1.25 ppm to 10 ppm (Piercey et al., 2017).

Unlike antibiotics, no critical concentrations for disinfectants have been defined to classify bacteria as disinfectant susceptible, intermediate or resistant. The determination of a resistance breakpoint is often arbitrarily defined. Several authors consider bacteria resistant if their MIC values are at least two to four times higher than those found

Table 3
Surface coverage (%) values in the observation field (14.161 μm^2) obtained for the biofilms by four *L. monocytogenes* strains after exposure for 10 min to different concentrations of sodium hypochlorite or benzalkonium chloride.

Treatment (10 min) with:		*Listeria monocytogenes* (strain)			
		LM2	LM6	LM7	LM12
No compound (control)		99.99 ± 0.01$^{a}_{a}$	99.99 ± 0.01$^{a}_{a}$	93.95 ± 6.48$^{b}_{ab}$	97.99 ± 0.37$^{a}_{a}$
Sodium hypochlorite (SHY)	0.5MIC	99.43 ± 0.87$^{a}_{a}$	99.97 ± 0.01$^{a}_{a}$	99.61 ± 0.58$^{a}_{a}$	99.99 ± 0.00$^{a}_{a}$
	1MIC	95.94 ± 5.44$^{ab}_{a}$	99.72 ± 0.32$^{a}_{a}$	55.62 ± 10.12$^{c}_{cd}$	82.86 ± 9.17$^{b}_{b}$
	1.5MIC	76.53 ± 2.38$^{a}_{ab}$	99.97 ± 0.04$^{b}_{a}$	72.86 ± 0.79$^{a}_{ac}$	59.90 ± 3.77$^{c}_{c}$
Benzalkonium chloride (BZK)	0.5MIC	62.18 ± 30.30$^{a}_{bc}$	94.53 ± 3.71$^{a}_{a}$	63.44 ± 33.29$^{a}_{bcd}$	76.45 ± 19.92$^{a}_{b}$
	1MIC	83.21 ± 9.34$^{a}_{ac}$	50.36 ± 6.05$^{b}_{b}$	41.31 ± 17.14$^{b}_{cd}$	80.05 ± 12.13$^{a}_{b}$
	1.5MIC	50.17 ± 39.65$^{a}_{b}$	31.33 ± 9.96$^{a}_{c}$	37.00 ± 36.46$^{a}_{d}$	21.21 ± 3.29$^{a}_{d}$

Data (mean ± STD) are the average of nine determinations. Means in the same row with no superscript letters in common are significantly different ($P < 0.05$). Means in the same column with no subscript letters in common are significantly different ($P < 0.05$). Surface coverage is calculated as percentage of green or red labelled pixels in the observation field (512 × 512 pixels).

Table 4
Maximum thickness (μm) values obtained for the biofilms by four *L. monocytogenes* strains after exposure for 10 min to different concentrations of sodium hypochlorite or benzalkonium chloride.

Treatment (10 min) with:		*Listeria monocytogenes* (strain)			
		LM2	LM6	LM7	LM12
No compound (control)		$31.23 \pm 2.07^{a}_{a}$	$23.76 \pm 0.52^{b}_{a}$	$22.28 \pm 2.71^{b}_{a}$	$16.10 \pm 0.24^{c}_{ab}$
Sodium hypochlorite (SHY)	0.5MIC	$21.01 \pm 2.00^{a}_{b}$	$21.23 \pm 0.58^{a}_{ab}$	$20.17 \pm 2.04^{a}_{ab}$	$25.25 \pm 1.52^{b}_{c}$
	1MIC	$17.56 \pm 2.52^{a}_{bc}$	$17.35 \pm 1.53^{a}_{bc}$	$11.23 \pm 1.15^{b}_{cd}$	$15.10 \pm 0.08^{a}_{a}$
	1.5MIC	$15.67 \pm 3.79^{ab}_{bc}$	$17.62 \pm 1.56^{a}_{bc}$	$9.25 \pm 0.57^{c}_{c}$	$12.50 \pm 0.50^{bc}_{d}$
Benzalkonium chloride (BZK)	0.5MIC	$18.87 \pm 2.08^{a}_{bc}$	$20.24 \pm 5.46^{a}_{ab}$	$16.40 \pm 3.93^{a}_{be}$	$16.43 \pm 2.41^{a}_{ab}$
	1MIC	$20.57 \pm 6.03^{a}_{b}$	$14.28 \pm 0.56^{b}_{c}$	$14.00 \pm 1.63^{b}_{d}$	$17.53 \pm 0.58^{ab}_{b}$
	1.5MIC	$13.50 \pm 2.12^{a}_{c}$	$14.29 \pm 1.55^{a}_{c}$	$12.68 \pm 1.08^{a}_{cde}$	$14.57 \pm 0.48^{a}_{a}$

Data (mean ± STD) are the average of nine determinations. Means in the same row with no superscript letters in common are significantly different ($P < 0.05$). Means in the same column with no subscript letters in common are significantly different ($P < 0.05$).

Table 5
Roughness values obtained for the biofilms by four *L. monocytogenes* strains after exposure for 10 min to different concentrations of sodium hypochlorite or benzalkonium chloride.

Treatment (10 min) with:		*Listeria monocytogenes* (strain)			
		LM2	LM6	LM7	LM12
No compound (control)		$0.1462 \pm 0.0669^{a}_{a}$	$0.1039 \pm 0.0009^{a}_{a}$	$0.3267 \pm 0.1453^{b}_{ab}$	$0.2818 \pm 0.0125^{b}_{a}$
Sodium hypochlorite (SHY)	0.5MIC	$0.2231 \pm 0.0604^{a}_{a}$	$0.1876 \pm 0.0080^{ab}_{b}$	$0.2005 \pm 0.0450^{a}_{a}$	$0.1137 \pm 0.0204^{b}_{b}$
	1MIC	$0.3743 \pm 0.1194^{ab}_{b}$	$0.2650 \pm 0.0752^{a}_{a}$	$0.4910 \pm 0.0359^{b}_{bc}$	$0.4386 \pm 0.0614^{b}_{c}$
	1.5MIC	$0.5126 \pm 0.0032^{a}_{bc}$	$0.2067 \pm 0.0253^{b}_{b}$	$0.4298 \pm 0.0038^{c}_{bc}$	$0.4947 \pm 0.0069^{a}_{c}$
Benzalkonium chloride (BZK)	0.5MIC	$0.5095 \pm 0.1411^{a}_{bc}$	$0.4702 \pm 0.0539^{a}_{d}$	$0.4712 \pm 0.1399^{a}_{bc}$	$0.4295 \pm 0.1823^{a}_{c}$
	1MIC	$0.5403 \pm 0.0355^{a}_{c}$	$0.5433 \pm 0.0057^{a}_{e}$	$0.5823 \pm 0.0743^{a}_{c}$	$0.4198 \pm 0.0577^{b}_{c}$
	1.5MIC	$0.5233 \pm 0.1039^{a}_{bc}$	$0.5784 \pm 0.0056^{a}_{e}$	$0.5333 \pm 0.0915^{a}_{c}$	$0.6213 \pm 0.0193^{a}_{d}$

Data (mean ± STD) are the average of nine determinations. Means in the same row with no superscript letters in common are significantly different ($P < 0.05$). Means in the same column with no subscript letters in common are significantly different ($P < 0.05$).

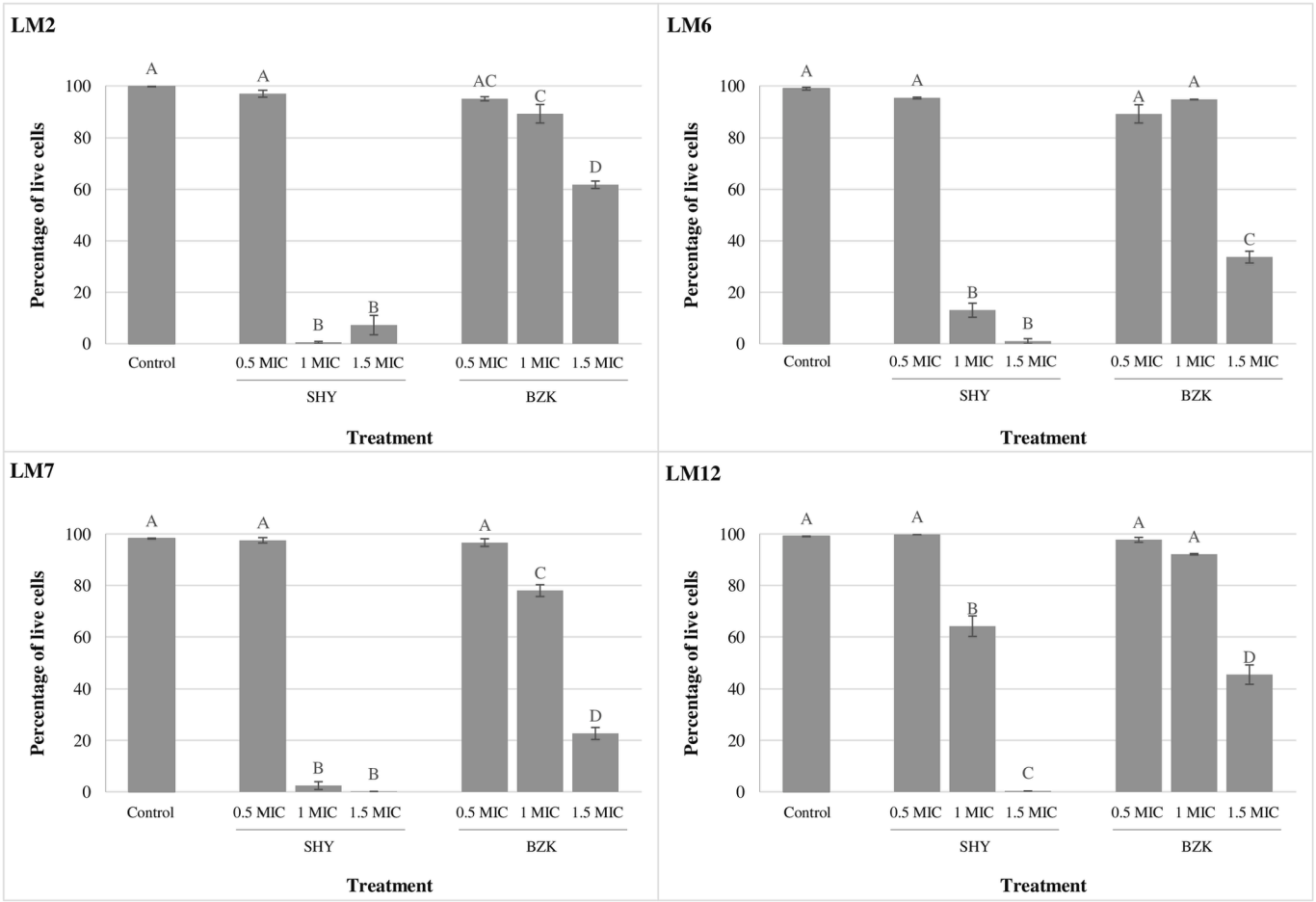

Fig. 2. Percentage of live cells in the biofilms of four *Listeria monocytogenes* strains before treatment (control) or after treatment for 10 min with different concentrations of sodium hypochlorite (SHY) or benzalkonium chloride (BZK). Bars in the same graphic with no letters in common are significantly different ($P < 0.05$). Data are the mean ± STD of nine determinations.

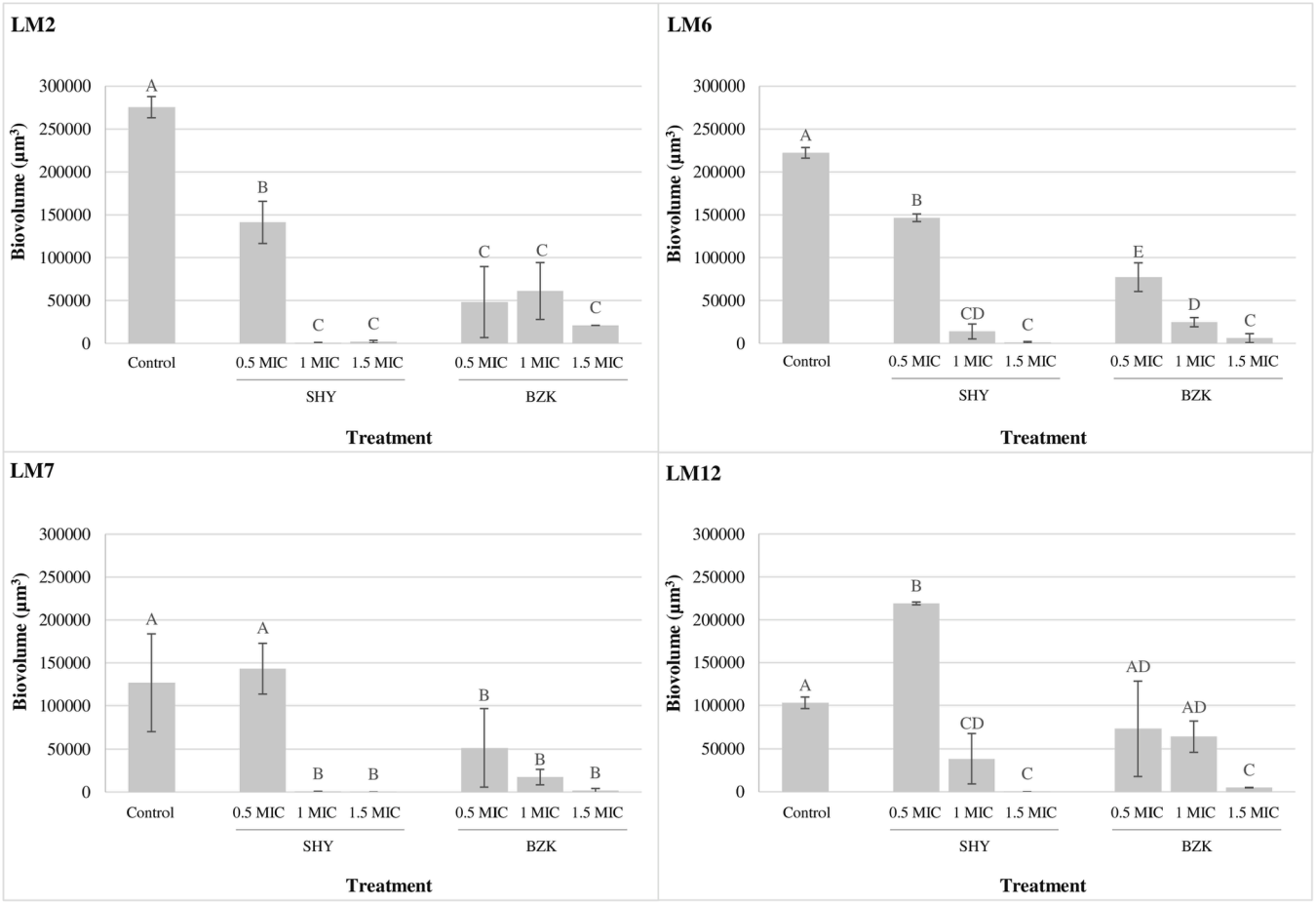

Fig. 3. Biovolume of live cells in the biofilms of four *Listeria monocytogenes* strains before treatment (control) or after treatment for 10 min with different concentrations of sodium hypochlorite (SHY) or benzalkonium chloride (BZK). Bars in the same graphic with no letters in common are significantly different ($P < 0.05$). Data are the mean ± STD of nine determinations.

in the most susceptible strains (Soumet et al., 2005). According to this criterion, two populations of strains have been identified in the present study: susceptible strains to BZK, with a MIC value of 3 ppm, and resistant strains to BZK, with MIC values ≥ 9 ppm. This breakpoint is in agreement with the results of Lemaître et al. (1998), Soumet et al. (2005) and Piercey et al. (2017), who held that strains with MIC values over 8 ppm, 7.5 ppm, and 7.5 ppm, respectively, were resistant to BZK.

In the present study, MIC and MBC values were below the recommended concentrations for SHY (800 ppm–2000 ppm of free chlorine; Norwood and Gilmour, 2000; Henriques and Fraqueza, 2017) and QAC (1000 ppm to 5000 ppm; Tamburro et al., 2015; Poimenidou et al., 2016; Henriques and Fraqueza, 2017) formulations. However, the level of exposure to disinfectants in food processing plants is dependent upon the sites where bacterial cells may shelter. Strains can encounter low concentrations of biocides in some circumstances (e.g. improper use, inappropriate storage or excessive amounts of organic matter, known to inactivate several biocides) (Capita et al., 2014), and it is very difficult to quantify concentration of biocides present at different locations at food processing facilities. In such scenario, SHY and BZK were tested so as to gain a better understanding of the susceptibility of planktonic and sessile *L. monocytogenes* cells to low concentrations of commonly used biocides, which will allow further optimization of strategies to control this major problem for the food industry.

4.2. Ability of L. monocytogenes to form biofilm

Control of *L. monocytogenes* in food processing environments can be a major challenge, and biofilm formation is one aspect of this problem. Confocal laser scanning microscopy (CLSM) enables three-dimensional optical imaging of biofilms using specific markers. CLSM together with quantitative image analyses permits the automatic determination of structural parameters of biofilms (e.g. biovolume, surface coverage, thickness or roughness) and of cellular viability by means of numerical data. This approach allows for the statistical analysis and comparison of biofilms under different conditions (e.g. different sanitizing treatments).

The microscopic observations performed for *L. monocytogenes* biofilms showed a dense three-dimensional structure, a result coincident with the reports of other authors (Borucki et al., 2003). The ability of *L. monocytogenes* to form moderate to strong biofilms has been observed previously by other authors after 24–48 h of incubation under static conditions on stainless steel (Rieu et al., 2008), glass (Bridier et al., 2010) or plastic, including polystyrene (Chavant et al., 2002; Torlak and Sert, 2013; Guilbaud et al., 2015). This is a worrying finding, because biofilms facilitate the persistence of this pathogen in food industries causing contamination of food during processing, thus representing a danger to public health.

The intraspecific diversity in the biomass of the biofilms formed by *L. monocytogenes* observed in this study is in accordance with findings from other research where quantitative methods were used to evaluate biofilm formation (Borucki et al., 2003; Di Bonaventura et al., 2008; Kadam et al., 2013; Guilbaud et al., 2015; Mosquera-Fernández et al., 2016; Henriques and Fraqueza, 2017).

4.3. Effect of SHY and BZK on the structural parameters and viability of biofilms formed by L. monocytogenes

To be useful against biofilms, disinfectants must possess either bactericidal activity or a removal capacity. The effect of treatment (10 min) with SHY or BZK at different concentrations (0.5 MIC, 1MIC or 1.5MIC) on 24-h-old *L. monocytogenes* preformed biofilms was evaluated. It was found that treatment with SHY (1MIC or 1.5MIC) or BZK (0.5MIC, 1MIC or 1.5MIC) decreased ($P < 0.05$) the number of live bacteria in biofilms. These results indicated that these substances have *anti*-biofilm properties at low concentrations. However bacterial eradication was not achieved.

In this study, SHY at concentrations of 1MIC or 1.5MIC decreased cell viability by more than 90% in comparison with non-treated biofilms, this suggesting that SHY has a mechanism of action on *L. monocytogenes* that would be mainly related to damaging membrane integrity, as previously reported for different microbial groups (Capita et al., 2014). On the other hand, the results demonstrated that BZK was able to remove most biofilm mass but reduced cellular viability only to a lesser extent. These results suggest that BZK was able to induce considerable biofilm detachment of life cells. This is a worrying result, because detached fragments of the biofilm could potentially colonize other surfaces. After treatment with BZK, more than 50% of the cells in biofilms were viable (Fig. 2). It is possible that the strong removal efficacy of BZK may have prevented the detection of more dead cells in CLSI images of BZK treated biofilms.

5. Conclusions

Listeria monocytogenes poses a significant safety challenge within the food industry. The effects of different concentrations and types of food-grade disinfectants against this bacterium must be discovered in order to achieve further optimization of strategies to control this major problem in food processing environments. This study highlights the differences between *L. monocytogenes* strains in respect of susceptibilities to BZK of planktonic cells. *L. monocytogenes* can adhere to, and produce dense biofilm on, polystyrene, a material commonly used in food processing environments. With concentrations up to one and a half times higher than the lowest concentration that achieved complete inhibition of growth of planktonic cells (1.5MIC), neither disinfectant achieved complete eradication of biofilms. However, treatment with biocides resulted in a reduction of the viability of biofilm structures (marked in the case of SHY). In addition, the biomass of biofilms decreased significantly after exposure to biocides (especially BZK). This fact implies that detachment of live cells from biofilms treated with low concentrations of disinfectants, mainly BZK, may occur, leading to a potential spread of the live bacteria within the food processing plant and subsequent food contamination, which is a matter for concern. These results may contribute to a better understanding of the behaviour and survival of *L. monocytogenes* in the presence of two sanitizers commonly used on food premises.

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