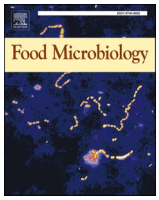
Food Microbiology 112 (2023) 104210

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm



Estimation by flow cytometry of percentages of survival of *Listeria monocytogenes* cells treated with tetracycline, with or without prior exposure to several biocides

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ARTICLE INFO

Keywords:
Listeria monocytogenes
Antibiotic resistance
Disinfectants
Flow cytometry

ABSTRACT

In certain circumstances, disinfectants are used at sublethal concentrations. The aim of this research work was to determine whether contact of *Listeria monocytogenes* NCTC 11994 with subinhibitory concentrations of three disinfectants widely used in food processing environments and in the health-care system, benzalkonium chloride (BZK), sodium hypochlorite (SHY) and peracetic acid (PAA), can cause the adaptation of the strain to the biocides and increase its resistance to tetracycline (TE). The minimum inhibitory concentrations (MIC; ppm) were 2.0 (BZK), 3500.0 (SHY) and 1050.0 (PAA). On exposure to increasing subinhibitory concentrations of the biocides, the maximum concentrations (ppm) of the compounds that allowed the strain to grow were (ppm) 8.5 (BZK), 3935.5 (SHY) and 1125.0 (PAA). Both the control cells (non-exposed) and the cells that had been in contact with low doses of biocides were treated with different concentrations of TE (0 ppm, 250 ppm, 500 ppm, 750 ppm, 1000 ppm and 1250 ppm) for 24, 48 and 72 h, and the survival percentages determined using flow cytometry, following dying with SYTO 9 and propidium iodide. The cells previously exposed to PAA presented higher survival percentages ($P < 0.05$) than the rest of the cells for most of the concentrations of TE and treatment times trialled. These results are worrying because TE is sometimes used to treat listeriosis, highlighting the importance of avoiding the use of disinfectant at subinhibitory doses. Furthermore, the findings suggest that flow cytometry is a fast and simple technique to obtain quantitative data on bacterial resistance to antibiotics.

1. Introduction

Listeria monocytogenes is the aetiological agent for human listeriosis, a food-borne infection that is especially serious in certain risk groups, particularly so-called YOPIs, in other words the young, the old, pregnant women and the immunocompromised (Schuchat et al., 1999). Each year the United States sees approximately 1600 cases of invasive listeriosis (the incidence rate is 0.26 cases per 100,000 inhabitants), and around 260 cases end in death (CDC, 2022). In the European Union a total of 1876 cases of invasive listeriosis were confirmed in 2020 (0.42 cases per 100,000 inhabitants), associated with a lethality rate of 13.0%, the highest among all diseases transmitted in foodstuffs (EFSA and ECDC, 2021).

L. monocytogenes presents a strong capacity to resist various adverse environmental factors, being able to grow in the presence of 10% of NaCl, and varying pH levels, tolerating a pH range from 4.1 to 9.6. Moreover, this bacterium is capable of growth over a wide temperature spectrum, running from 1 °C to 45 °C, and is a facultative anaerobe (Rocourt and Buchrieser, 2007). These characteristics favour the persistence of *L. monocytogenes* in food-processing environments, where the bacterium may linger for long periods (Ortiz et al., 2016).

In disinfecting surfaces and equipments both in food industries and in the health-care systems various different biocides are used, such as benzalkonium chloride (BZK), sodium hypochlorite (SHY) and peracetic acid (PAA) (Cadena et al., 2019; Capita et al., 2019; Querido et al., 2019; Pereira and Tagkopoulos, 2019). For these disinfectants to be effective, they must be used in appropriate concentrations. However, the use of sub-inhibitory concentrations of biocides is relatively frequent in food-processing environments. This may be the outcome of an incorrect calculation of concentrations, of an uneven distribution of the

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https://doi.org/10.1016/j.fm.2022.104210
Received 14 February 2022; Received in revised form 21 November 2022; Accepted 20 December 2022
Available online 23 December 2022

Table 1
Minimum inhibitory concentrations (MICs) and maximum biocide concentrations permitting microbial growth of *L. monocytogenes* NCTC 11994 after adaptation.

Biocide	MIC (ppm)[a]	Adaptation (ppm)[b]
BZK[c]	2.0	8.5
SHY[d]	3500.0	3935.5
PAA[e]	1050.0	1125.0

[a] Minimum inhibitory concentration.
[b] Maximum biocide concentration permitting microbial growth after various passes through gradually increasing concentrations of biocides.
[c] Benzalkonium chloride.
[d] Sodium hypochlorite.
[e] Peracetic acid.

substances used, or of the presence of excessive amounts of organic matter, which inactivates some biocide chemicals (for example, chlorinated compounds), among other causes (Alonso-Hernando et al., 2009, 2011; Buzón-Durán et al., 2017; Capita et al., 2014, 2017; Molina--González et al., 2014).

Antibiotic resistance is a major public health problem, and its repercussions are growing. Infections caused by resistant bacteria are very hard to treat, since many regularly used antibiotics are ruled out as therapeutic options. This difficulty in treating them involves increased costs for health systems, apart from their considerable impact in terms of morbidity and mortality (Dadgostar, 2019). It has been suggested that there is a direct relationship between reduced susceptibility to biocides and to antibiotics, since the mechanisms contributing to both are similar, for instance, changes in the permeability of cell membranes or the synthesis of efflux pumps (Alonso-Calleja et al., 2015; Capita et al., 2014). Thus, in previous research involving enterobacteria (*Escherichia coli, Cronobacter sakazakii, Yersinia enterocolitica* and *Salmonella enterica*) it was observed that exposure to sub-inhibitory doses of disinfectants led to adaptation by bacterial populations to biocides, simultaneously modifying the pattern of resistance to antibiotics of these strains (Alonso-Hernando et al., 2009; Capita et al., 2014, 2017, 2019; Molina-González et al., 2014).

Determination of the degree of susceptibility to a given antibiotic using methods dependent on culturing is slow, and this may lead to the prescription of an unsuitable antibiotic because of the urgent need for treatment (WHO World Health Organization, 2014). Several alternatives to such profiles of microbial susceptibility, or "antibiograms", are based on an evaluation of given indicators of cell viability, such as the integrity of cellular membranes, employing fluorescent dyes (e. g. SYTO 9 and propidium iodide, PI).

SYTO 9 is a dye that gives a green fluorescence (510 nm–540 nm) to bacterial nucleic acids and can penetrate both intact and damaged bacterial cells. After incubation with SYTO 9 practically all bacteria emit green fluorescence when excited with blue light, this permitting a count of total cells. In contrast, PI cannot get inside bacteria with intact membranes. Hence, the emission of red fluorescence (620 nm–650 nm) is associated only with permeabilized bacteria (Freire et al., 2015). When both dyes are present in the same cell, emissions from SYTO 9 diminish as a consequence of displacement of one dye by the other, an outcome of the greater affinity of PI for nucleic acids. The usefulness of SYTO 9 and PI in evaluating bacterial viability has been previously demonstrated (Capita et al., 2019; Robertson et al., 2019; Rodríguez-Melcón et al., 2018, 2019a, 2019b).

Fluorescence may be ascertained by means of flow cytometry, a technique that can provide information about the sensitivity to antibiotics of a microbial population (Jarzembowski et al., 2008; March--Rosselló, 2017; Robertson et al., 2019). It has been indicated that SYTO 9 and PI for detecting cell viability of *L. monocytogenes* by flow cytometry offers rapid and reproducible staining, with high-intensity fluorescence and sensitivity, combined with a good correlation of results with the number of colony-forming units (Jacobsen et al., 1997).

The objectives of this research work were: 1) to know the percentages of *L. monocytogenes* cells surviving different treatments (concentrations and exposure times) with tetracycline, 2) to determine the influence of previous exposure to sub-inhibitory concentrations of several biocides on the survival percentages of *L. monocytogenes* to tetracycline, and 3) to investigate the usefulness of the flow cytometry technique for these purposes.

2. Material and methods

2.1. Strain and disinfectants

L. monocytogenes NCTC 11994 (serotype 4b) was used. This strain was selected because it was isolated from a food sample (soft cheese) and belongs to the serovar 4b, which is the most frequently associated with both outbreaks and individual cases of human listeriosis (Farber and Peterkin, 1991; Ragon et al., 2008). Three disinfectants were tested: benzalkonium chloride (BZK, product number B6295, Sigma-Aldrich Co., St. Louis, Missouri, United States) sodium hypochlorite (containing 10% of free chlorine, NaClO, SHY, product number 425044, Sigma-Aldrich) and peracetic acid (PAA, product number 77240, Sigma-Aldrich). The solutions of each substance were prepared under aseptic conditions in sterile distilled water just prior to the start of each experiment.

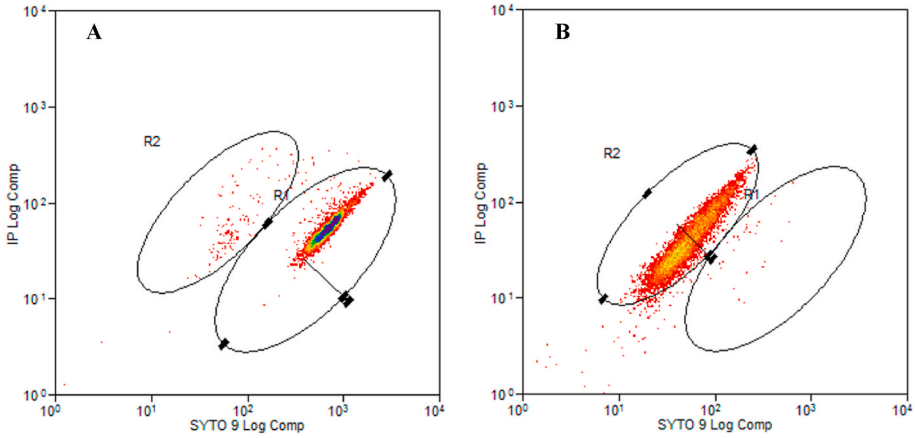

Fig. 1. Dot plots of controls: A, control of live cells of *L. monocytogenes* NCTC 11994, with 98.27% viability in region 1 (R1); B, control of dead cells of *L. monocytogenes* NCTC 11994, with 98.15% inactivation in region 2 (R2).

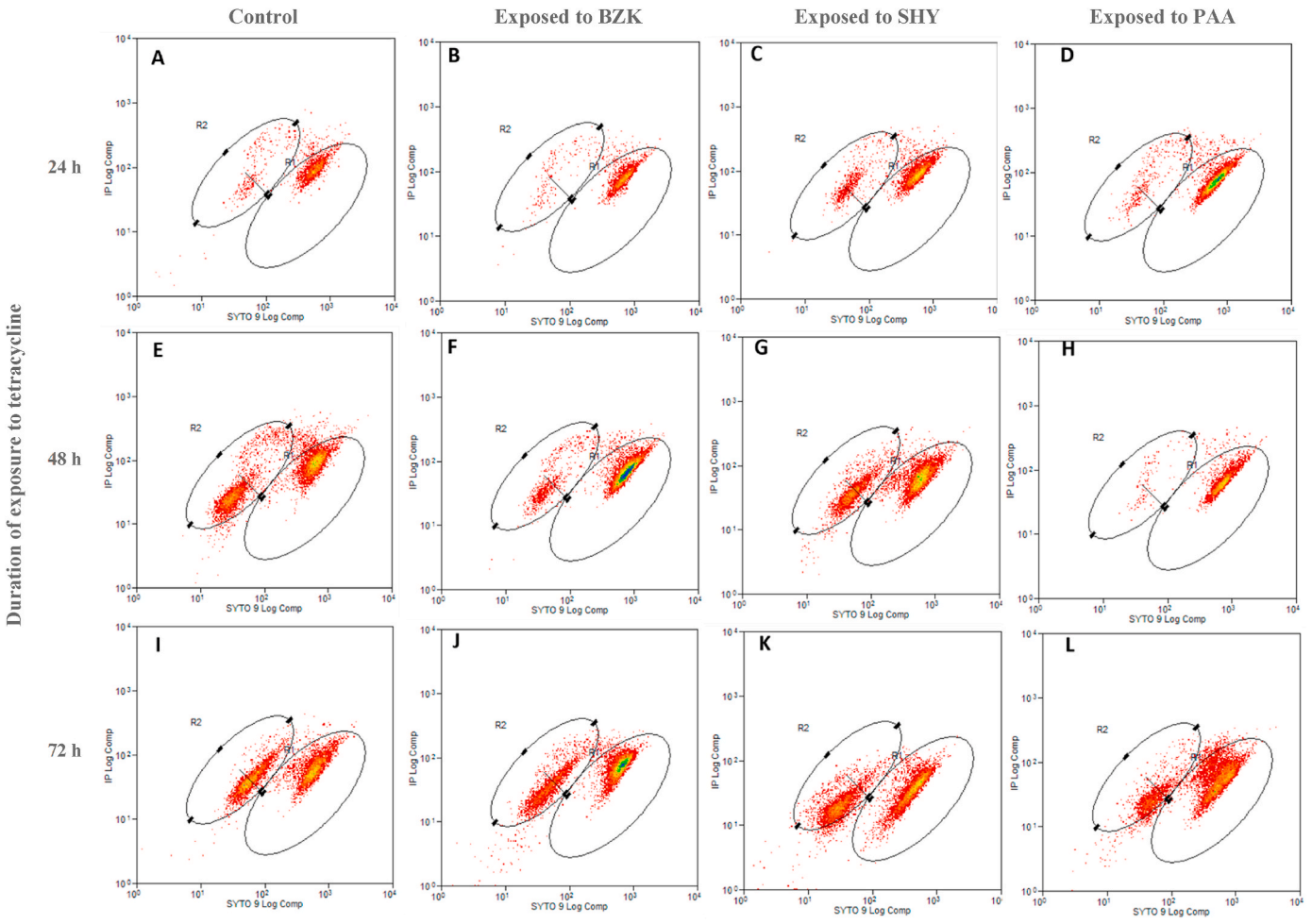

Fig. 2. Dot plot of live (region 1, R1) and dead (region 2, R2) cells of *L. monocytogenes* NCTC 11994 after prior exposure to benzalkonium chloride (BZK: B, F, J), sodium hypochlorite (SHY: C, G, K) or peracetic acid (PAA: D, H, L) or not exposed to biocides (control: A, E, I) and treated with 0 ppm tetracycline at 30 °C for 24 h (A, B, C, D), 48 h (E, F, G, H), or 72 h (I, J, K, L).

2.2. Calculation of minimum inhibitory concentrations (MICs)

The MICs for *L. monocytogenes* were calculated in accordance with the protocol previously described by Capita et al. (2014), with very slight modifications. Five colonies were taken from plates of tryptone soya agar (TSA; product number CM0131, Oxoid Ltd., Hampshire, England). These were inoculated into tubes containing 9 ml of tryptone soya broth (TSB; product number CM0129, Oxoid) and incubated at 37 °C for 24 h so as to obtain a final concentration in the tubes of approximately 5×10^8 cfu/ml, the concentration being confirmed by plating. Three decimal dilutions of this inoculum were made in TSB. For the experiment, polystyrene microtitre plates with one hundred wells were used (Oy Growth Curves Ab Ltd., Helsinki, Finland). These were filled with 20 μl of the disinfectant solution (BZK, SHY or PAA at the various different concentrations) plus 180 μl of the third dilution of the strain in TSB, so as to obtain a final concentration in each well of approximately 5×10^5 cfu/ml. Use was also made of controls, positive with 200 μl of the third dilution of the culture in TSB and negative with 200 μl of TSB. Bacterial growth was determined by measuring optical density at 420 nm–580 nm ($OD_{420\text{-}580}$) in a Bioscreen C MRB (Oy Growth Curves Ab). The MIC was set at the minimum concentration of biocide needed to prevent bacterial growth ($OD_{420\text{-}580} \leq 0.200$) after 48 h of incubation at 37 °C.

2.3. Exposure to increasing sub-inhibitory concentrations of biocides

The procedure was similar to that employed to calculate MICs. In the wells of a microtitre plate (Oy Growth Curves Ab) 180 μl of the third dilution of culture broth (approximately 5×10^5 cfu/ml) were deposited along with 20 μl of biocide, so as to achieve a concentration of MIC/2 in a given well. When growth was observed by measuring the $OD_{420\text{-}580}$ in a Bioscreen C MRB after 48 h of incubation at 37 °C, a 20 μl extract of the suspension was transferred to the next well, which contained 160 μl of TSB (Oxoid) and 20 μl of disinfectant solution. The biocide concentration in this well was thus one and a half times greater than that in the previous well. This procedure was repeated until no growth was observed after 72 h of incubation at 37 °C. The suspension from the last well in which growth was seen was plated on TSA (Oxoid) with the corresponding biocides (half the maximum concentration of biocide that allowed microbial growth was added to TSA). In the case of strains not exposed to disinfectants, TSA was used without biocides. After incubation at 37 °C for 48 h, plates were stored at 4 °C ± 1 °C for a maximum of one week.

2.4. Exposure to tetracycline

The first step was to inoculate five colonies from each of the TSA plates (control, BZK, SHY and PAA) into 10 ml of TSB (for strains that had not previously been exposed to disinfectants) or of TSB with the

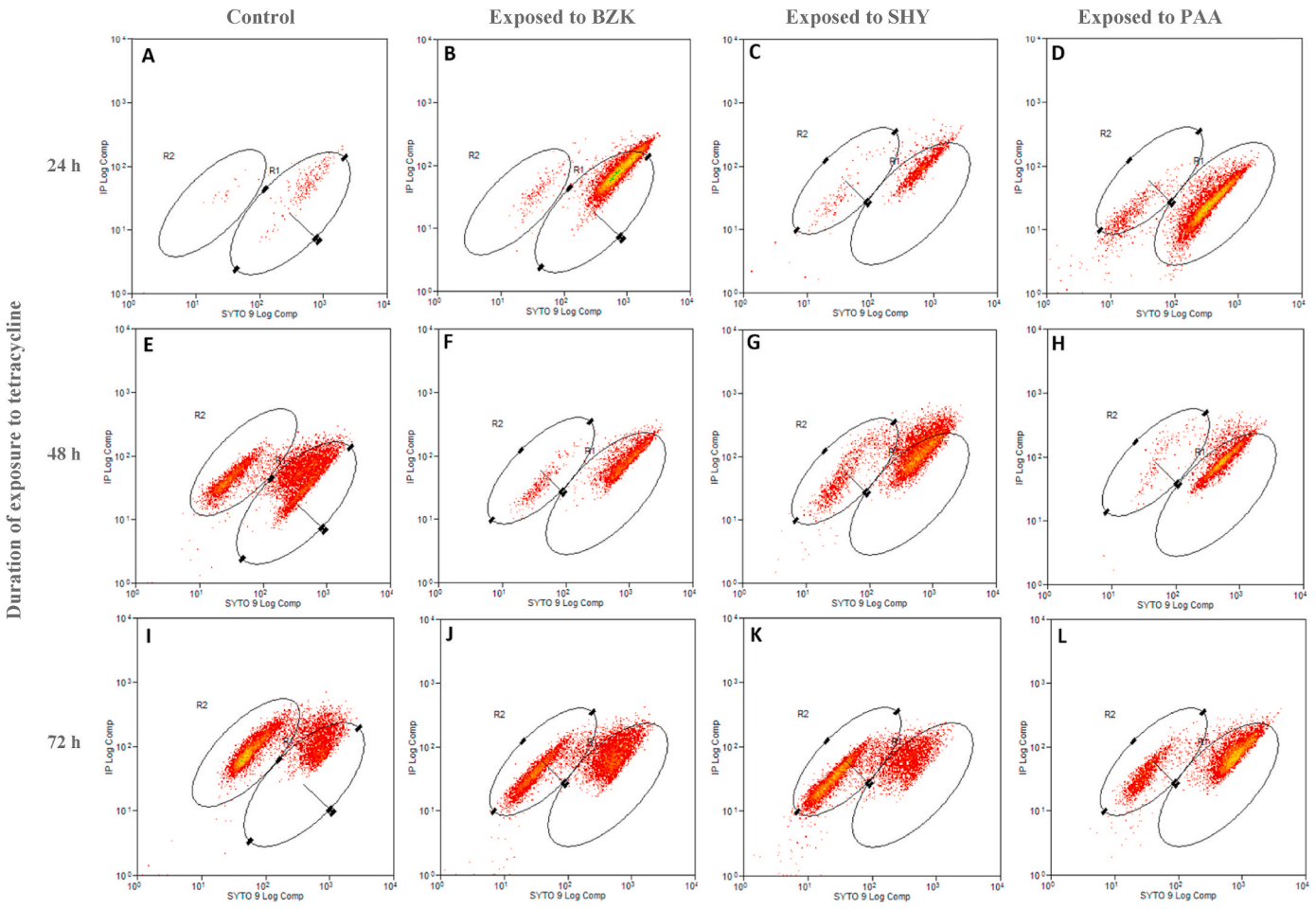

Fig. 3. Dot plot of live (region 1, R1) and dead (region 2, R2) cells of *L. monocytogenes* NCTC 11994 after prior exposure to benzalkonium chloride (BZK: B, F, J), sodium hypochlorite (SHY: C, G, K) or peracetic acid (PAA: D, H, L) or not exposed to biocides (control: A, E, I) and treated with 250 ppm tetracycline at 30 °C for 24 h (A, B, C, D), 48 h (E, F, G, H), or 72 h (I, J, K, L).

relevant quantity of biocide if strains were previously been exposed to disinfectants (half the maximum concentration of biocide permitting microbial growth in each instance was used). Thereafter, strains were incubated for 24 h at 37 °C to achieve a final concentration in the tube of approximately 5×10^8 cfu/ml. Once this period had elapsed, the contents of the glass tubes were transferred to sterile plastic tubes and centrifuged for 20 min at 3200×*g* and a temperature of 4 °C. The supernatant was removed and 10 ml of 0.85% sodium chloride (NaCl, product number 31434, Sigma-Aldrich) was added. Using sterile Falcon tubes, 900 μl of the strain suspended with sodium chloride were mixed with 100 μl of an appropriate aqueous solution of tetracycline (TE, product number TE4062, Sigma-Aldrich). The concentrations tested were 0 ppm, 250 ppm, 500 ppm, 750 ppm, 1000 ppm and 1250 ppm of tetracycline. Exposure to the antibiotic was at 30 °C for 24 h, 48 h or 72 h. Tetracycline was tested because this drug has been used for years in the treatment of listeriosis in both animals and human beings when first-line drugs cannot be applied (e. g. patients with allergy to beta-lactams) (Buchner and Schneierson, 1968; Clark, 2013).

2.5. Staining of samples

A total of 987 μl of 0.85% NaCl solution, 1.5 μl of SYTO 9 (a 3.34 mM solution in dimethyl sulphoxide or DMSO) and 1.5 μl propidium iodide (PI, 20 mM solution in DMSO) from a BacLight Viability Kit (product number 10266712, Invitrogen, Carlsbad, California, United States) were added to sterile plastic tubes. Thereafter, 10 μl of the strain subjected to the various treatments with TE was added, so that the final concentration in the tubes, including viable and inactivated cells, was approximately 10^6 cfu/ml. To verify measurements, positive and negative controls for the samples were undertaken with known percentages of bacteria in the exponential growth phase (18–24 h of incubation). Positive controls were composed of untreated live cells, and negative of inactivated cells heated to 90 °C for 20 min. Both types of control were stained with the two dyes mentioned. Similarly, there were controls of unstained live cells, serving to determine and calibrate bacterial autofluorescence. After addition of the dyes, all tubes were incubated at 30 °C for 20 min in darkness to enhance their penetration into the bacterial cells.

2.6. Visualization and quantification by flow cytometry

Analysis of samples was performed with a CyAn ADP flow cytometer (Beckman Coulter, Brea, California, United States) using the Summit V4.4.00 software. The 488 nm excitation source was an argon laser, used with two detectors: FL1 (530/40, 545 digital light processing or DLP) for green fluorescence, and FL3 (613/20, 640 DLP) for red fluorescence. The voltage for the acquisition parameters in the case of the fluorochromes was 560 v for FL1 (corresponding to SYTO 9) and 597 v for FL3 (corresponding to PI).

Side scatter (SSC) is defined as light dispersed at an angle of 90° from the incident light of the laser. It is associated with the rugosity, or wrinkled nature, of the surface of elements inside cells, and indicates

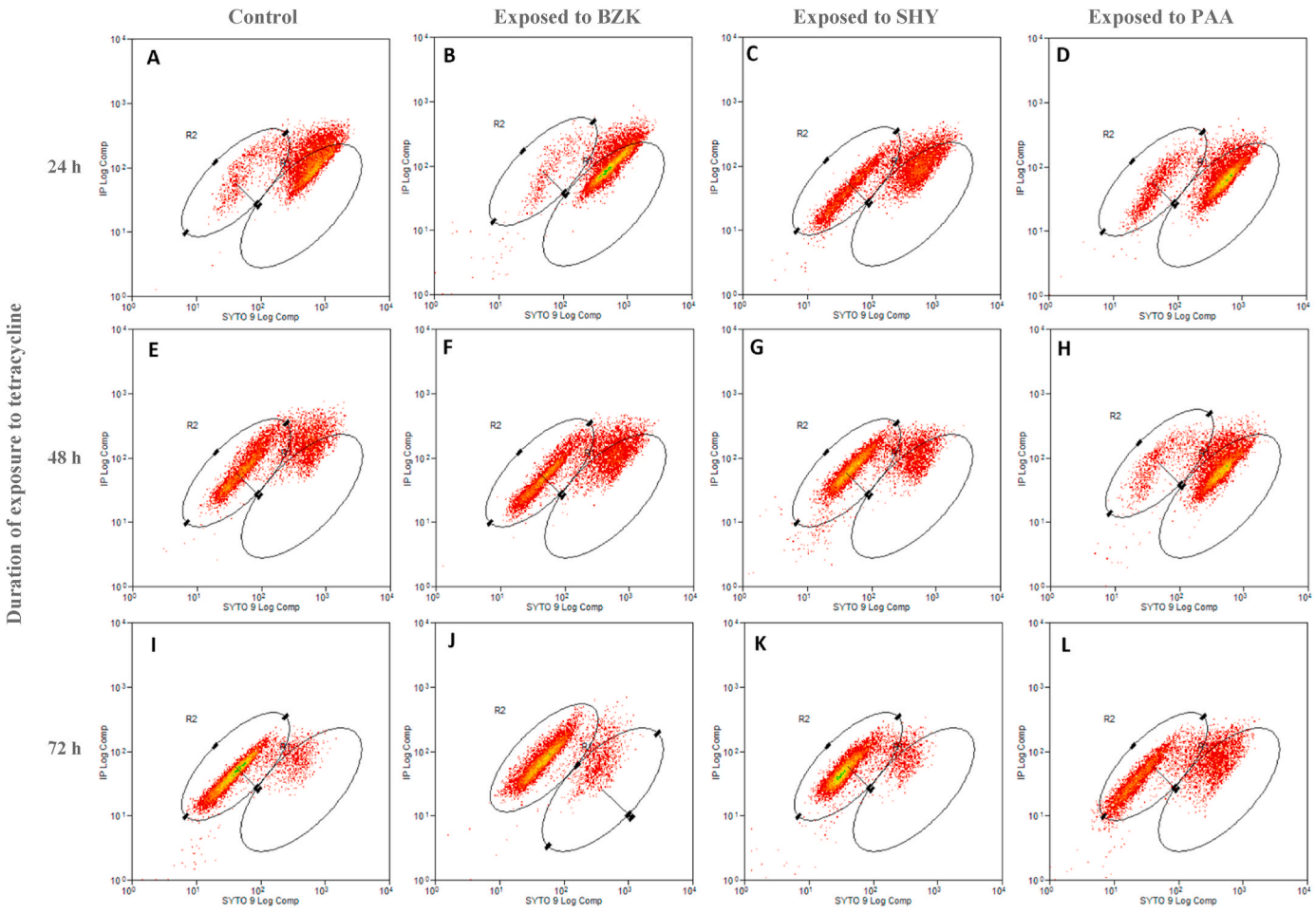

Fig. 4. Dot plot of live (region 1, R1) and dead (region 2, R2) cells of *L. monocytogenes* NCTC 11994 after prior exposure to benzalkonium chloride (BZK: B, F, J), sodium hypochlorite (SHY: C, G, K) or peracetic acid (PAA: D, H, L) or not exposed to biocides (control: A, E, I) and treated with 500 ppm tetracycline at 30 °C for 24 h (A, B, C, D), 48 h (E, F, G, H), or 72 h (I, J, K, L).

relative internal complexity or granularity; the greater this is, the greater will be the complexity recorded by the SSC detector. For the side scatter detector, it was 752 v. For all values, a predetermined gain of 1.0 was set. A threshold value, or cut-off point, the percentage at which an event would be detected by the cytometer, was set at 0.01%. The parameter for frontal dispersion, or forward scatter (FSC), is related to the relative size of cells; this is the light that a bacterial cell does not allow to be transmitted directly ahead, and is linked to the surface area or size of the cell. A gain of 50.0% was set for FSC. Thereafter, a region or gate was established so as to discriminate from background noise in the zone of interest where the cell population was to be found, on the basis of cell complexity (SSC, on the ordinate, or Y axis) as against cell size (FSC, on the abscissa, or X axis), together with the emission of fluorescence. All the analyses were performed at slow speed, using a stop condition of 10,000 events, or cells, in the region selected, with 100,000 events as the maximum. The parameters were expressed on a logarithmic scale as a dot plot.

2.7. Statistical analysis

Quantitative parameters for bacteria were compared by analysis of variance (ANOVA) techniques, with means separated using Duncan's Multiple Range Test. Significant differences were established for a probability level of 95% ($P < 0.05$). Data processing was performed using the software package Statistica® 8.0 (StatSoft Ltd., Tulsa, Oklahoma, United States).

3. Results

3.1. Minimum inhibitory concentrations (MICs) and adaptation of cultures to growing concentrations of biocides

The values of MICs for BZK, SHY and PAA for the *L. monocytogenes* strain trialled are presented in Table 1. This also shows the values for maximum concentrations of biocides allowing microbial growth after successive passes in TSB with growing concentrations of these substances.

SHY was the biocide requiring the highest concentrations to inhibit growth of *L. monocytogenes* after 48 h of incubation at 37 °C: 3500.0 ppm, equivalent to 350 ppm of free chlorine. After various passes through gradually increasing concentrations of this biocide, the strain managed to grow with a concentration of 3935.5 ppm of SHY. The MIC for peracetic acid (PAA) was 1050.0 ppm, with a final growth concentration of 1125.0 ppm in the strain exposed to subinhibitory concentrations of the compound. BZK produced inhibition of bacterial growth at the lowest concentration, 2.0 ppm. However, it was also the biocide to which the strain adapted most, achieving growth at concentrations of 8.5 ppm (a value 4.25 higher than the original MIC).

3.2. Percentages of survival of cells of L. monocytogenes with and without prior exposure to biocides when treated with tetracycline

Prior to analysis of samples, a set of controls were established to

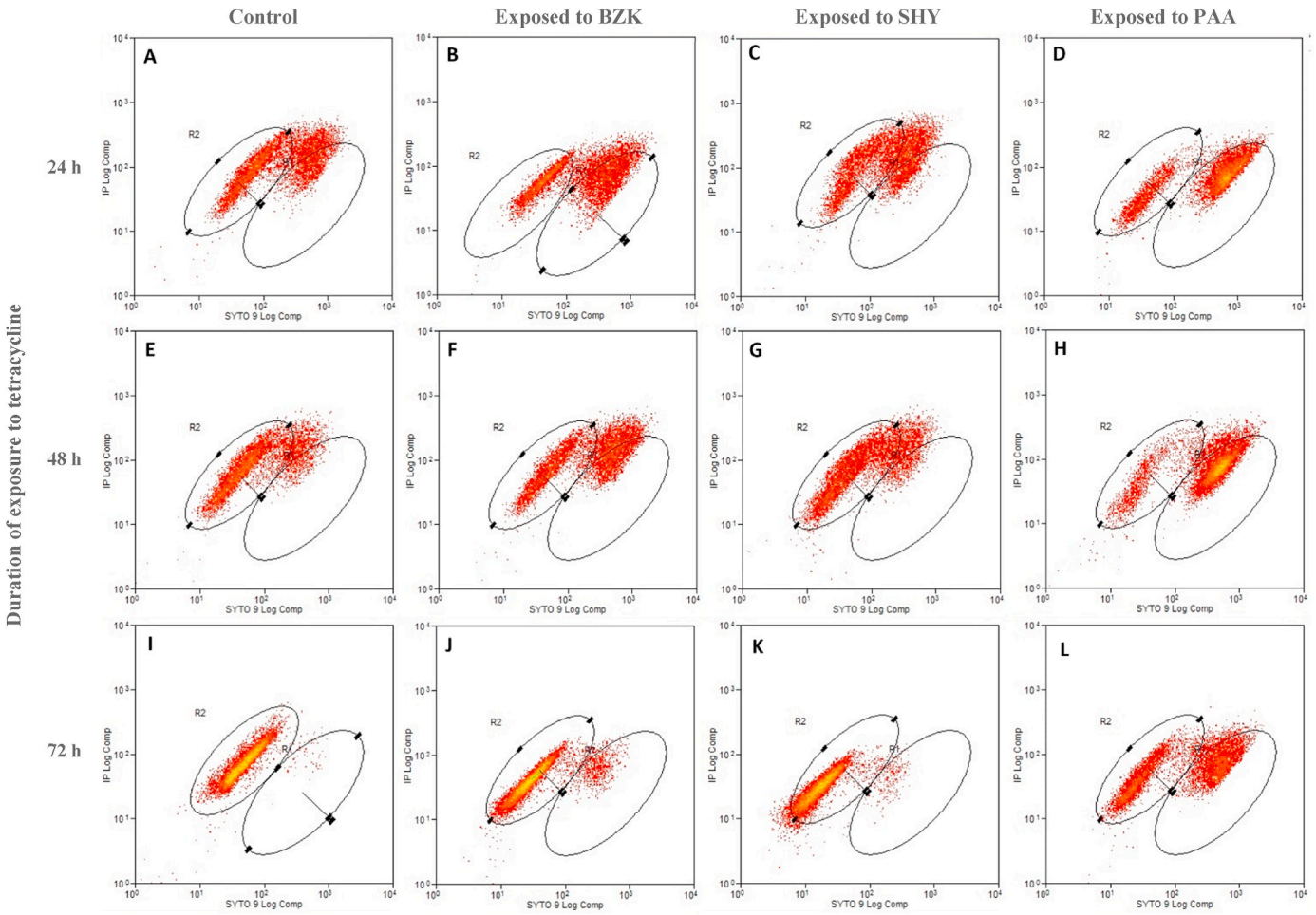

Fig. 5. Dot plot of live (region 1, R1) and dead (region 2, R2) cells of *L. monocytogenes* NCTC 11994 after prior exposure to benzalkonium chloride (BZK: B, F, J), sodium hypochlorite (SHY: C, G, K) or peracetic acid (PAA: D, H, L) or not exposed to biocides (control: A, E, I) and treated with 750 ppm tetracycline at 30 °C for 24 h (A, B, C, D), 48 h (E, F, G, H), or 72 h (I, J, K, L).

allow optimization of acquisition protocols for results. In this previous phase, use was made of positive and negative controls, the former being cultures of *L. monocytogenes* incubated for 24 h at 37 °C, and the latter cultures of the same species subjected to 90 °C for 20 min. Both types of cultures were stained with the fluorescent dyes SYTO 9 and PI, so that the cell content of each sample could be verified, as could its percentage of viability (Fig. 1).

Flow cytometry data allowed estimation of the impact of tetracycline (TE) on microbial viability and cell damage. Figs. 2–7 show examples of the dot plots for various groups of cells after exposure to TE. Subsequent to double marking of cells with the dyes SYTO 9 and PI, it proved possible to identify two clear subpopulations: in region 1 (R1) were to be found live cells marked with SYTO 9, as established by trials undertaken with the viability controls, and in region 2 (R2) were the dead or unviable cells, stained with PI. As the time of exposure to TE or the concentrations of this antibiotic grew, the density of the population detected in R1 shrank, progressively moving into R2, owing to a decrease in SYTO 9 fluorescence and replacement of this dye by PI.

It should be noted that there is a subpopulation outside both these regions in some dot plots. This subpopulation may reflect doubly stained cells, viable but harmed or damaged, in which PI did not succeed in displacing all the SYTO 9 dye because the membrane was not sufficiently impaired to allow all the dye to reach the cell's DNA. Such subpopulations were not taken into account in performing the statistical analysis of the data.

Table 2 and Fig. 8 show the average percentage of live cells after exposure to different concentrations of tetracycline (0 ppm, 250 ppm, 500 ppm, 750 ppm, 1000 ppm and 1250 ppm) over each of the periods of incubation used in the experiment (24 h, 48 h and 72 h), both among unexposed control cells and those previously exposed to growing sub-inhibitory concentrations of the biocides. The percentage of surviving cells ranged between 0.58% ± 0.43% for control cultures treated with 750 ppm of TE for 72 h, and 95.54% ± 3.37% for cultures previously exposed to BZK and incubated for 24 h in the absence of any TE.

When no TE was present, higher ($P < 0.05$) percentages of cell viability were observed after 72 and 48 h of incubation in cultures with prior exposure to BZK (between 71.64% ± 16.59% and 85.85% ± 9.54% of live cells) and to PAA (ranging from 89.10% ± 9.64% to 93.42% ± 2.70%) than in cultures with no exposure (control; between 51.15% ± 15.67% and 71.65% ± 10.83%) or exposed to SHY (with a range running from 52.53% ± 8.18% to 70.27% ± 10.68%). When cells were treated with tetracycline, prior exposure to PAA increased the percentage of bacterial survival relative to control cultures or those exposed to SHY, after challenge with tetracycline over 72 h (with 250 ppm TE), 48 and 72 h (with 500 ppm TE), 24, 48 and 72 h (with 750 ppm and 1000 ppm TE), or 24 and 72 h (with 1250 ppm TE), as shown in Fig. 8. The percentage of survival seen in cultures with prior exposure to BZK fell in an intermediate position, between the control cultures or those exposed to SHY, and the cultures subjected to PAA.

Both in the case of control cells and in that of cells previously

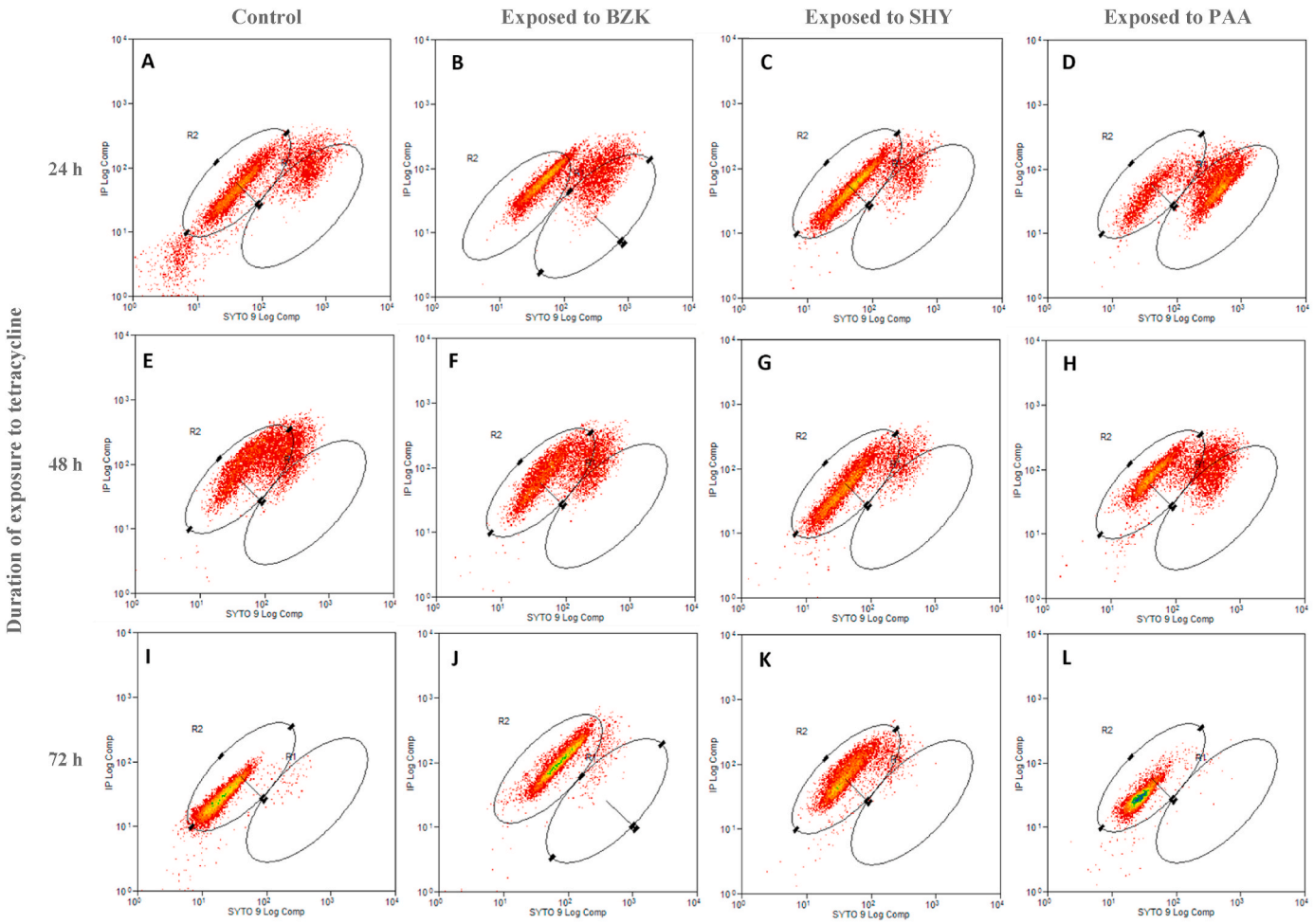

Fig. 6. Dot plot of live (region 1, R1) and dead (region 2, R2) cells of *L. monocytogenes* NCTC 11994 after prior exposure to benzalkonium chloride (BZK: B, F, J), sodium hypochlorite (SHY: C, G, K) or peracetic acid (PAA: D, H, L) or not exposed to biocides (control: A, E, I) and treated with 1000 ppm tetracycline at 30 °C for 24 h (A, B, C, D), 48 h (E, F, G, H), or 72 h (I, J, K, L).

exposed to biocides, the concentration of TE impacted the percentage of surviving cells. As might be expected, the lowest values were observed at the highest concentrations tested. Similarly, the longer the period of treatment with TE, the lower the concentration of cells surviving for most of the conditions investigated (Table 2).

4. Discussion

4.1. Minimum inhibitory concentrations (MICs) and adaptation of cultures to increasing concentrations of biocides

In the study being reported here, BZK produced inhibition of the growth of *L. monocytogenes* at 2.0 ppm, the lowest concentration among the biocides utilized. Prior work with *L. monocytogenes* (Rodríguez-Melcón et al., 2019a) recorded MICs of 3–14 ppm for BZK, similar to the values in the current research. Likewise, BZK has proved to be a very effective biocide against other microorganisms. A MIC of 2 ppm was noted for methicillin-resistant *Staphylococcus aureus*, or MRSA, by Buzón-Durán et al. (2017), a figure of 8 ppm for *Salmonella enterica* serotype Typhimurium by Capita et al. (2017), and values between 15 ppm and 20 ppm for *Cronobacter sakazakii* and *Yersinia enterocolitica* by Capita et al. (2019).

The MIC for SHY observed in the present investigation also fell within the range of values previously recorded for strains of other Gram-positive bacteria. Thus, Buzón-Durán et al. (2017) and Rodríguez-Melcón et al. (2019b), determined the MIC for strains of methicillin-resistant *Staphylococcus aureus* (MRSA) and for *L. monocytogenes* of the serotype 1/2a, and obtained values of 5000 ppm and 3000 ppm, respectively. As for PAA, the figures for MICs observed by Capita et al. (2019) were between 1200 ppm and 1275 ppm for Gram-negative bacteria, specifically *Cronobacter sakazakii* and *Yersinia enterocolitica*, and these values coincide with the findings from the current study. However, other authors (Alonso-Hernando et al., 2011) obtained MIC figures between 100 ppm and 110 ppm for *L. monocytogenes* when using peroxyacids. The variations in these results may be due to the fact that not all microorganisms present the same susceptibility to different biocides. Moreover, there are certain differences in the composition of biocides, since some are made up of several chemicals (Capita et al., 2019).

In the present research it was found that after exposure to increasing sub-inhibitory concentrations of BZK, *L. monocytogenes* was able to grow with concentrations of the biocide 4.25 times higher than the MIC. This makes it plain there was strong adaptation to this disinfectant, more than to any other of the biocides studied. The results of work by Capita et al. (2019) corroborate these findings, since in their experiments *C. sakazakii* after adaptation to BZK was capable of growth in the presence of 3.8 times the MIC of this compound. Some researchers, such as Sidhu et al. (2004), have noted that lactic acid bacteria with prior tolerance to BZK adapt more easily and to greater concentrations of this disinfectant than bacteria sensitive to it. It should be pointed out that a

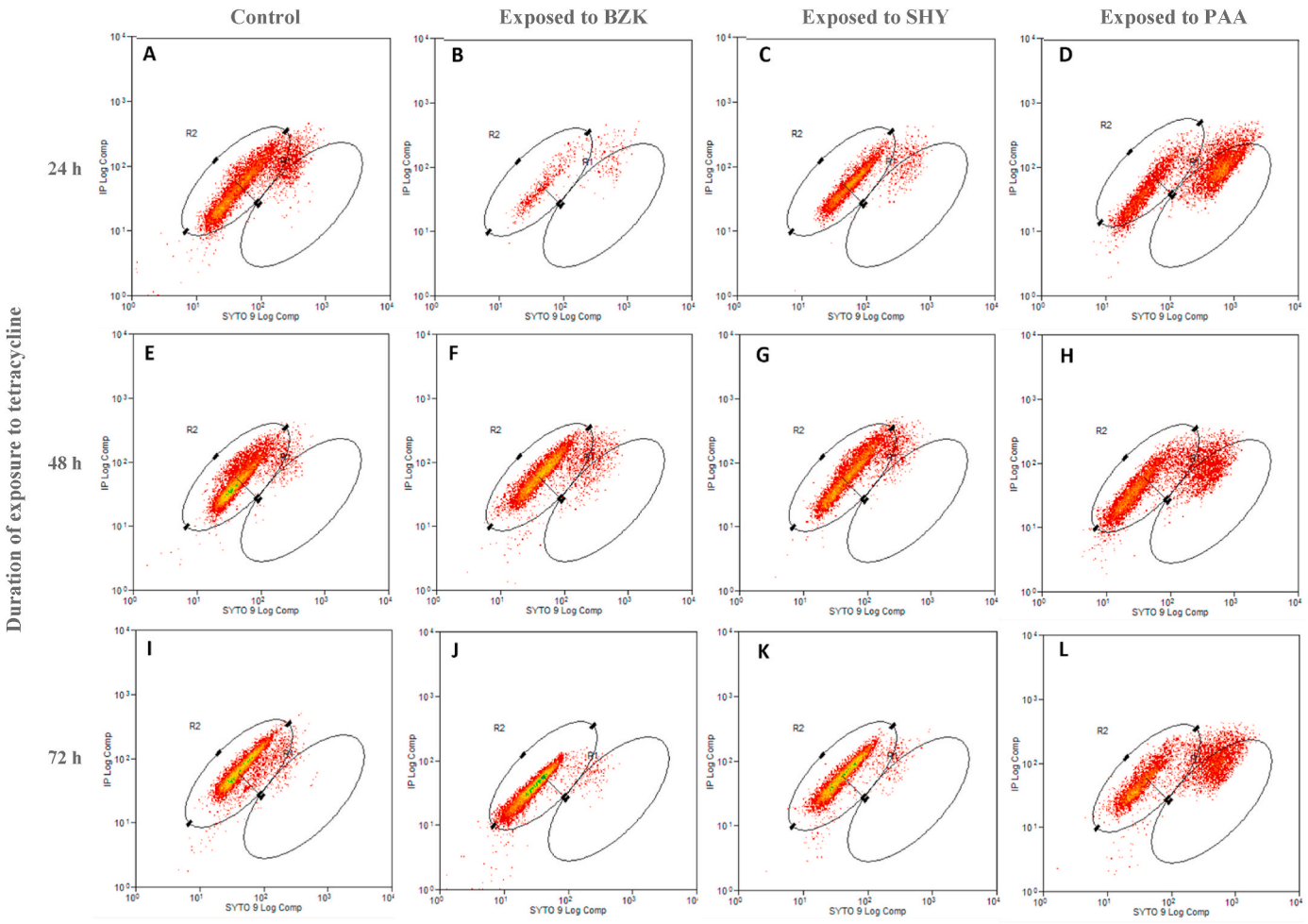

Fig. 7. Dot plot of live (region 1, R1) and dead (region 2, R2) cells of *L. monocytogenes* NCTC 11994 after prior exposure to benzalkonium chloride (BZK: B, F, J), sodium hypochlorite (SHY: C, G, K) or peracetic acid (PAA: D, H, L) or not exposed to biocides (control: A, E, I) and treated with 1250 ppm tetracycline at 30 °C for 24 h (A, B, C, D), 48 h (E, F, G, H), or 72 h (I, J, K, L).

reduction in susceptibility to biocides after repeated exposure of strains to sub-inhibitory concentrations of such substances has previously been observed in different groups of bacteria (Alonso-Hernando et al., 2009; Capita et al., 2014, 2017, 2019; Molina-González et al., 2014).

After several passes through culture medium with increasing sub-inhibitory concentrations of biocides, it was found that the maximum concentration of SHY permitting bacterial growth (3935.5 ppm) was higher than the MIC. These results coincide with what was observed by Capita et al. (2019), who noted that *Yersinia enterocolitica* adapted to SHY when confronted with limited doses of the biocide. Specifically, after exposure to increasing sub-inhibitory concentrations of SHY, the strain became capable of growing in the presence of a concentration of the substance 2.53 times higher than the MIC. However, for PAA these authors observed very slight adaptation, a result that is in agreement with those reported here.

4.2. *Percentages of survival of Listeria monocytogenes cells treated with tetracycline, with or without prior exposure to various biocides*

It would appear that the present research is the first to use flow cytometry to determine the effect of exposure to increasing sub-inhibitory concentrations of three disinfectants (BZK, SHY, PAA) upon the susceptibility of *L. monocytogenes* to antibiotics, specifically tetracycline. It proved possible to observe a clear separation between two specific subpopulations, of live and of inactivated bacteria, by flow cytometry using SYTO 9 and PI dyes together in samples. In this way, it was feasible to establish the regions delimiting these subpopulations in the samples treated with TE on the basis of controls undertaken. The fluorescence signals for the two dyes were acceptable, as percentages higher than 98% were recorded for both subpopulations (Fig. 1), this ensuring that SYTO 9 would have a high intensity only in R1, and PI only in R2. Figs. 2–7 show a clear drop in SYTO 9 fluorescence in control cells challenged with tetracycline (plots A, E, I), as there was virtually no fluorescence detected in R1. Stiefel et al. (2015) demonstrated that displacement of SYTO 9 by PI is effective, as they observed a signal from this dye nine times weaker in dead cells of *S. aureus* than in living, which provides backing for the results being reported here.

Other authors (Freire et al., 2015; Jarzembowski et al., 2008) have also shown the usefulness of flow cytometry in studying bacterial permeability induced by antibiotics. Novo et al. (2000) used flow cytometry techniques to demonstrate that concentrations of tetracycline at 4 μg/ml permeabilized the membranes of 50% of cells of *S. aureus* and *Micrococcus luteus* stained with ThermoFisher TO-PRO-3 stain (red fluorescence from inactivated cells) and $DiOC_2(3)$ (3,3′-diethyloxacarbocyanine iodide; green fluorescence from live cells) after 4 h of exposure to the drug. Their results do not coincide with those presented here, in which higher concentrations of TE and longer exposure times were needed to eliminate 50% of the population of *L. monocytogenes* (Table 2). These differences between studies might be explained, at least in part, by intraspecific differences (Novo et al., 2000).

Table 2
Percentage (%) of cells surviving treatment with tetracycline (at different concentrations and exposure times) in strains of *L. monocytogenes* NCTC 11994 with and without (control) prior exposure to sub-inhibitory concentrations of biocides.

Cultures previously exposed to[a]	Duration (h) of treatment with tetracycline	Concentration of tetracycline (ppm)					
		0	250	500	750	1000	1250
Control (non-exposed)	**24**	$92.79 \pm 5.57^{c}_{c}$	$86.74 \pm 19.87^{c}_{b}$	$71.16 \pm 21.78^{bc}_{b}$	$44.32 \pm 31.13^{ab}_{b}$	$35.31 \pm 32.96^{ab}_{b}$	$21.04 \pm 34.23^{a}_{a}$
	48	$71.65 \pm 10.83^{b}_{b}$	$64.79 \pm 29.91^{b}_{ab}$	$25.87 \pm 25.11^{a}_{a}$	$11.19 \pm 14.72^{a}_{a}$	$5.55 \pm 4.67^{a}_{a}$	$3.01 \pm 2.96^{a}_{a}$
	72	$51.15 \pm 15.67^{a}_{a}$	$34.04 \pm 22.41^{b}_{a}$	$3.83 \pm 3.95^{a}_{a}$	$0.58 \pm 0.43^{a}_{a}$	$0.82 \pm 0.48^{a}_{a}$	$1.34 \pm 1.17^{a}_{a}$
BZK[b]	**24**	$95.54 \pm 3.37^{c}_{b}$	$91.92 \pm 7.09^{c}_{b}$	$77.00 \pm 15.74^{c}_{b}$	$51.59 \pm 17.84^{b}_{b}$	$35.01 \pm 21.28^{b}_{b}$	$12.87 \pm 9.62^{a}_{b}$
	48	$85.85 \pm 9.54^{c}_{a}$	$87.01 \pm 6.04^{c}_{a}$	$43.21 \pm 29.63^{b}_{a}$	$25.29 \pm 20.09^{ab}_{a}$	$19.72 \pm 18.81^{ab}_{ab}$	$5.81 \pm 7.26^{a}_{ab}$
	72	$71.64 \pm 16.59^{b}_{a}$	$56.54 \pm 32.73^{b}_{a}$	$20.85 \pm 23.18^{a}_{a}$	$5.30 \pm 5.04^{a}_{a}$	$1.44 \pm 0.55^{a}_{a}$	$1.33 \pm 1.09^{a}_{a}$
SHY[c]	**24**	$91.30 \pm 6.16^{c}_{c}$	$88.22 \pm 9.68^{c}_{b}$	$63.30 \pm 27.02^{b}_{b}$	$23.73 \pm 18.93^{a}_{b}$	$15.85 \pm 14.62^{a}_{b}$	$5.30 \pm 2.07^{a}_{b}$
	48	$70.27 \pm 10.68^{b}_{b}$	$72.47 \pm 13.44^{c}_{b}$	$25.03 \pm 17.12^{b}_{a}$	$9.51 \pm 10.89^{a}_{ab}$	$5.37 \pm 4.72^{a}_{ab}$	$1.78 \pm 0.86^{a}_{a}$
	72	$52.53 \pm 8.18^{b}_{a}$	$45.83 \pm 27.07^{b}_{a}$	$7.45 \pm 3.92^{a}_{a}$	$2.30 \pm 1.32^{a}_{a}$	$1.15 \pm 0.43^{a}_{a}$	$0.93 \pm 0.67^{a}_{a}$
PAA[d]	**24**	$93.83 \pm 6.79^{b}_{a}$	$90.59 \pm 6.42^{b}_{a}$	$72.05 \pm 27.72^{ab}_{a}$	$66.07 \pm 31.01^{ab}_{a}$	$65.78 \pm 29.80^{ab}_{b}$	$52.65 \pm 34.65^{a}_{a}$
	48	$93.42 \pm 2.70^{c}_{a}$	$87.26 \pm 4.20^{c}_{a}$	$65.69 \pm 26.84^{bc}_{a}$	$49.80 \pm 30.54^{ab}_{a}$	$41.49 \pm 28.42^{ab}_{ab}$	$22.61 \pm 28.75^{a}_{a}$
	72	$89.10 \pm 9.64^{a}_{a}$	$80.68 \pm 17.40^{c}_{a}$	$49.21 \pm 37.09^{b}_{a}$	$31.04 \pm 20.88^{ab}_{a}$	$16.08 \pm 18.89^{a}_{a}$	$16.31 \pm 19.08^{a}_{a}$

Values (n = 5) in the same row sharing a superscript letter show no significant differences one from another ($P > 0.05$); values in the same column, where different time periods are compared for the same group of cells, sharing any subscript letter show no significant differences one from another ($P > 0.05$).

[a] Cultures were exposed to increasing sub-inhibitory concentrations of biocides until no further growth was observed.

[b] Benzalkonium chloride.

[c] Sodium hypochlorite.

[d] Peracetic acid.

The results shown in Figs. 2–8 and in Table 2 highlight clear changes in the resistance of the strain to tetracycline after exposure to sub-inhibitory doses of biocides. In the case of the control cells, not exposed to biocides, at the highest concentration investigated (1250 ppm) and after 24 h of exposure, the percentage of live cells observed was 21.04%. In contrast, in cultures adapted to PAA the survival percentage under the same conditions was 52.65%. Nevertheless, cells adapted to SHY presented results similar to those exposed to BZK, the first having 5.30% viability after 24 h of exposure to 1250 ppm of TE and the second 12.87%. According to Bansal et al. (2018), exposure to sub-lethal concentrations of SHY provokes oxidative stress related to changes in cell ultrastructure, such as cell wall thickness, membrane shape or modifications to cytoplasm in strains of *L. monocytogenes,* and consequently augments its vulnerability to the antibiotic.

It is of particular note that there was strong resistance to TE in cells previously exposed to PAA. Indeed, in the case of cells adapted to this disinfectant, a mortality close to 100% was not achieved with any of the concentrations or exposure times trialled. Such a result is worrying, because tetracycline can be used to treat listeriosis in patients allergic to beta-lactams (Olaimat et al., 2018; Wilson et al., 2018). It highlights the crucial need to use this disinfectant at appropriate concentrations. It should be noted that there have been few studies investigating links between adaptation to PAA and decreased sensitivity to antibiotics in strains of Gram-positive bacteria (Kampf, 2019). Hence, the outcomes of this research work are novel.

Some previously published work testing various different disinfectants and strains of bacteria has given varying perspectives on the appearance of resistance to antibiotics because of the use of biocides. Thus, Molina-González et al. (2014) and Randall et al. (2007) observed that using certain types of biocides may increase resistance in *Salmonella enterica* to a range of disinfectants and antibiotics. Other authors, such as Potenski et al. (2003), have pointed out that exposing Gram-negative bacteria to sub-lethal concentrations of additives, like acetic acid, may enhance resistance to multiple antibiotics, among them tetracycline. For their part, Romanova et al. (2006) studied the behaviour of strains of *L. monocytogenes* naturally sensitive to BZK. They found that after adaptation to BZK these strains presented cross-adaptation to other quaternary ammonium compounds with a similar mode of action, and to certain antibiotics, such as gentamicin and kanamycin. All this may be due to cross-resistance phenomena involving antibiotics and disinfectants, probably related to an increased expression of non-specific efflux pumps (Levy, 2002).

As indicated previously, some cases were observed in the dot plots outside the regions selected from Fig. 1. On similar lines, several authors (Paparella et al., 2008) have detected that when essential oils are used, three different subpopulations also appear: viable, dead and injured cells. These authors observed that the third population, of damaged cells, grows as the concentration of the substance increases.

5. Conclusions

Exposure to increasing sub-inhibitory concentrations of SHY and BZK triggered adaptation by the *L. monocytogenes* NCTC 11994 strain (serotype 4b) to biocides, especially in the case of BZK, with a more than four-fold increment in the concentration of the substance it was capable of resisting after adaptation. Moreover, contact with low doses of BZK and especially of PAA augmented the resistance of cells of this bacterium to tetracycline. The finding is worrying, since this is an antibiotic utilized to treat listeriosis as an alternative to beta-lactams. The results obtained point to the usefulness of the flow cytometry technique, through staining with SYTO 9 and PI, in studying antibiotic resistance of cells of *L. monocytogenes* subjected to various different conditions, its rapidity and simplicity being its outstanding features.

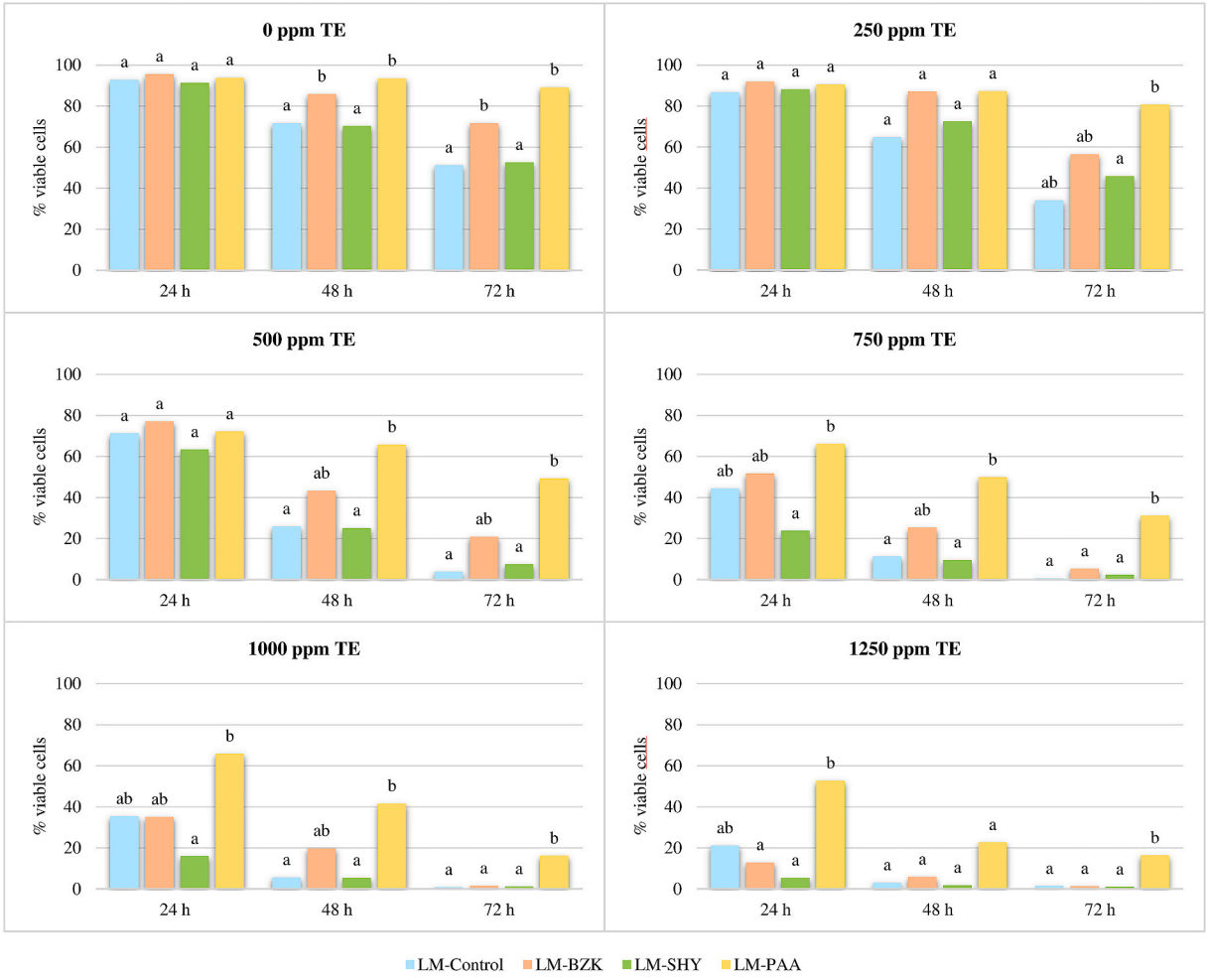

Fig. 8. Percentage of survival of *L. monocytogenes* (LM) exposed to benzalkonium chloride (BZK), sodium hypochlorite (SHY), peracetic acid (PAA), or unexposed (control) after treatment with different concentrations of tetracycline (TE, ppm) for 24, 48, o 72 h at 30 °C. Columns having the same tetracycline concentration and treatment time that share any letter show no significant differences one from another ($P > 0.05$).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This research was founded by the *Ministerio de Ciencia, Innovación y Universidades* (Spain, grant number RTI2018-098267-R-C33), the *Junta de Castilla y León* (*Consejería de Educación*, Spain, grant number LE018P20), and the *Universidad de León* (Spain, grant number 18BB282).

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