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Effect of low doses of biocides on the susceptibility of *Listeria monocytogenes* and *Salmonella enterica* to various antibiotics of clinical importance

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ABSTRACT

The use of subinhibitory concentrations of biocides in food processing environments requires special attention because it is related to potential increases in antibiotic resistance. In this study, we determined the effect of exposure to low doses of four biocides (sodium hypochlorite, SHY; benzalkonium chloride, BZK; peracetic acid, PAA; trisodium phosphate, TSP) on the resistance to 10 antibiotics and on the hydrophobicity of the cellular surface of a strain of *Listeria monocytogenes* serotype 1/2a (LM) and a strain of *Salmonella enterica* serotype Agona (SA), both of meat origin. The cultures were exposed at 37 °C in Mueller Hinton II cation-adjusted broth with 0.6% yeast extract (with 0.2% of laky horse blood added in the case of LM) to increasing concentrations of the biocides, starting with half the minimum inhibitory concentration (MIC/2) and incrementing by 1.5 times the concentration until growth was no longer observed, calculating the MIC of the antibiotics before (control cultures) and after exposure. After exposure to TSP, LM was able to grow in the presence of a concentration of the biocide 2.53 times higher than the MIC of unexposed cultures. No adaptation was observed for SHY, BZK or PAA. SA demonstrated adaptation to BZK (it tolerated a concentration 1.13 times higher than the MIC for the unadapted strain) and PAA (2.53 times). LM cultures presented increased resistance (from susceptibility to reduced susceptibility, from susceptibility to resistance, or from reduced susceptibility to resistance) to erythromycin (strains exposed to BZK, PAA and TSP) and fosfomycin (all compounds). Regarding SA, after exposure its resistance to cefoxitin (all compounds), gentamicin (all compounds), tetracycline (TSP), fosfomycin (SHY, BZK and TSP) and enrofloxacin (BZK, PAA and TSP) increased. The cell surface hydrophobicity (determined through the microbial adhesion to solvents -MATS- test) increased (LM exposed to PAA and TSP; SA exposed to BZK) or decreased (SA exposed to PAA) after contact with the biocides. These findings suggest that the use of biocides at subinhibitory concentrations can contribute to the increase in bacterial resistance to antibiotics, in addition to modifying the hydrophobicity of the cellular surface, which is related to the capacity of bacteria to form biofilm.

1. Introduction

Listeria monocytogenes is a very widespread zoonotic bacterium responsible for listeriosis, an infection affecting particularly certain risk groups, known as YOPIs (the young, the old, pregnant women and the immunocompromised). In the European Union, 2183 cases of invasive listeriosis (0.49 cases per 100,000 population) were confirmed in 2021. They were associated with a hospitalization rate of 96.5% and a lethality rate of 13.7%, both these figures being the highest among all food-borne illnesses (EFSA & ECDC, 2022). Treatment with antibiotics is usually necessary for invasive listeriosis (Rugna et al., 2021).

For its part, salmonellosis is the zoonosis linked to the largest number of outbreaks of food-borne disease in the European Union. It comes in second place only to campylobacteriosis in terms of the overall number of cases of human bacterial infection. The number of cases of salmonellosis confirmed in 2021 totalled 60,050, implying a notification rate of 15.7 per 100,000 inhabitants (EFSA & ECDC, 2022).

For some decades now, antibiotic resistance has been a worldwide preoccupation of the first order, since the difficulty of treating infections by resistant bacteria involves considerable expense for health systems, apart from their major impact in terms of morbidity and mortality. Despite control measures that have been introduced, this problem has continued to evolve (Capita & Alonso-Calleja, 2013). An increase in the prevalence of resistance to antibiotics has also been observed in recent

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years in isolates of *L. monocytogenes*. This species continues to be largely sensitive to clinically relevant antimicrobials, but more and more often presents reductions in susceptibility, particularly in strains isolated from food-production lines (Alonso-Hernando et al., 2012; Gómez et al., 2014; Komora et al., 2017).

The biocides used in the food industry at times are applied in sub-lethal concentrations. This can be an outcome, for instance, of an error in calculating the correct strength, inappropriate storage of products leading to a loss of effectiveness, low stability of the chemical substance, insufficient cleaning prior to disinfection, or of an uneven distribution of effective components (Capita et al., 2014; Frisón et al., 2015). In recent years, it has been pointed out that contact with low doses of biocides may trigger enhanced tolerance to biocides and greater resistance to antibiotics, since the mechanisms contributing to these circumstances are similar (Capita et al., 2019).

In previous work it has been demonstrated that changes in the hydrophobicity of the cell surface occurring after exposure to low doses of biocides may play a key role in decreased sensitivity of Gram-negative bacteria to antibiotics (Alonso-Calleja et al., 2015; Capita et al., 2014). Gaining an understanding of the response of microorganisms to conditions that may occur in food-processing environments would be of considerable help in developing effective disinfection strategies. It was in this context that the current work was undertaken, its aim being to determine the influence of exposure to low doses of various biocides widely used in the food industry upon the susceptibility to different antimicrobials and the hydrophobicity of the cell surface in strains of *L. monocytogenes* and *S. enterica*.

2. Material and methods

2.1. Strains of bacteria and biocides

Two bacterial strains were used: *Listeria monocytogenes* (LM; serotype 1/2a) and *Salmonella enterica* serotype Agona (SA), isolated from pork meat and chicken meat, respectively, and already available in our laboratory. Bacterial cultures were stored at −50 °C in tryptone soya broth (TSB, Oxoid Ltd., Hampshire, United Kingdom) with 20% (vol/vol) glycerol. Before each experiment, the bacteria were transferred to tubes of TSB (Oxoid) and incubated for the whole night at 37 °C. Thereafter, strains were cultured on plates of tryptone soya agar (TSA, Oxoid), kept in store at 4 °C.

Four biocides were tested, including sodium hypochlorite (NaClO, SHY, 10% of free chlorine, Sigma-Aldrich Co., St. Louis, Missouri, U.S.A., CAS number 7681-52-9), benzalkonium chloride (BZK, Sigma-Aldrich, CAS number 63449-41-2), peracetic acid (PAA, Sigma-Aldrich, CAS number 79-21-0) and trisodium phosphate (TSP, Merck, Darmstadt, Germany, CAS number 10101-89-0). The solutions of each biocide were prepared under aseptic conditions in sterile distilled water just prior to the start of each experiment.

2.2. Calculation of minimum inhibitory concentrations of biocides

Minimum inhibitory concentrations (MICs) were calculated with a method involving microdilution in broth in accordance with the guidelines from the Clinical and Laboratory Standards Institute, U.S.A. (CLSI, 2018). Five colonies of each strain were taken from plates coated with TSA (Oxoid) and inoculated into 9 ml of BBL™ Mueller Hinton II cation-adjusted broth (MHB, Becton Dickinson and Company, Sparks Glencoe, Maryland, U.S.A.) with 0.6% yeast extract (YE, Oxoid). For the incubation and corresponding dilutions of LM, 0.2% of laky horse blood (Thermo Fisher Scientific, Hampshire, United Kingdom) was added. The bacteria were incubated at 37 °C for 18–24 h. In the experiment, 100-well polystyrene microtitre plates (Oy Growth Curves Ab Ltd., Helsinki, Finland) were used. The wells were filled with 20 μl of the biocides, at a range of concentrations, together with 180 μl of inoculum at the appropriate dilution in MHB with YE, and with added blood in the case of LM. This was to ensure a final concentration in the well of approximately 10^5 cfu/ml. Negative controls (culture medium and solutions of biocides) and positive controls (inoculum) were used. The concentration of inoculum was confirmed by plating. Growth was determined by measuring the optical density ($OD_{420\text{-}580}$) in a Bioscreen C MRB (Oy Growth Curves Ab). MIC was set as the minimum concentration of biocide needed to prevent bacterial growth after 48 h of incubation. On the basis of previous trials, the growth limit was taken to be an $OD_{420\text{-}580}$ of 0.200 for strains not using blood (SA) and of 0.600 for those that did use it (LM).

2.3. Adaptation of strains to the biocides used

The experiment was performed in the same way as the determination of MICs, with slight modifications (Capita et al., 2014). The starting concentration of biocide in the microtitre plate was MIC/2. When growth was observed, a quantity of 20 μl of the suspension was transferred under sterile conditions to the next well, which contained 160 μl of culture broth (Mueller Hinton II cation-adjusted broth with 0.6% yeast extract, adding 0.2% of laky horse blood in the case of LM) and 20 μl of the solutions of biocides. This was calculated to ensure that each well held a concentration of biocide one and a half times greater than the preceding well. This procedure was repeated until no growth was detectable after 72 h of incubation at 37 °C. The suspension from the last well in which growth was observed was plated onto TSA together with the relevant biocide, with the TSA having added to it half the maximum concentration of this permitting microbial growth. Unexposed cells were grown in MHB + YE, with 0.2% of blood added in the case of *L. monocytogenes*, and transferred to plates of TSA without biocides. After incubation at 37 °C for 48 h, the agar plates were kept at 4 °C for a maximum of one week. For later stage of the experiment, biocides were added at half the maximum concentration permitting growth to tubes holding 9 ml of MHB + YE, and 0.2% of blood for *L. monocytogenes*, so as to promote the growth of bacterial cells exposed to biocides, and the same broth without biocides for the control cells. The tubes were inoculated with the strains kept on plates with TSA, if the cells were not previously exposed to biocides, or TSA plus biocides, if the cells were previously exposed to SHY, BZK, PAA or TSP. All groups of cultures were trialled simultaneously.

2.4. Calculation of minimum inhibitory concentrations of antibiotics

Using the method above-described for biocides, the minimum inhibitory concentrations (MICs) of ten antibiotics was determined for the strains of LM and SA with and without prior exposure to the various different biocides tested. Use was made of the following dehydrated antibiotics (Sigma-Aldrich): ampicillin (AMP), sodium cephalothin (KF), cefoxitin (FOX), erythromycin (E), chloramphenicol (C), gentamicin (CN), tetracycline hydrochloride (TE), vancomycin hydrochloride (VA), sodium fosfomycin (FOS) and enrofloxacin (ENR).

2.5. Determination of cell surface hydrophobicity (CSH)

Surface hydrophobicity of the microbial cells was determined through a microbial adhesion to solvents test (MATS) based on affinity to non-polar solvents (Capita et al., 2014). In the experiment, the bacteria were put into tubes containing 9 ml of cation-adjusted BBL™ Mueller Hinton II broth (MHB, Becton Dickinson and Company) with 0.6% yeast extract (YE, Oxoid). In the case of cells exposed to biocides, the broth contained half the maximum concentration of biocide allowing growth after adaptation, or half the MIC when cultures had not been able to adapt. The tubes utilized for LM contained 0.2% of laky horse blood (Thermo Fisher Scientific). Strains were incubated for 24 h at 37 °C. Thereafter, cells in the exponential growth phase were harvested by centrifuging at 4000 rpm (10 min at 4 °C) in sterile plastic tubes. Cells were rinsed twice with phosphate-buffered saline at a pH of 7.4 (PBS,

Merck). They were then centrifuged once more and re-suspended with an inoculating loop in sterile tubes under the same conditions as described above. Strains were incubated for 24 h at 37 °C, with an initial concentration in the tube of 10^5 cfu/ml, and a final concentration of approximately 10^9 cfu/ml. Once this time had elapsed, a dilution was carried out in such a way as to ensure the presence of 10^8 cfu/ml for both microorganisms, and harvested by centrifuging at 4000 rpm for 10 min at 4 °C in sterile plastic tubes. These cells were rinsed twice with PBS, centrifuged once again and re-suspended in 2.4 ml of a 150 mM solution of sodium chloride (NaCl) at a concentration of approximately 10^8 cfu/ml. Two samples of the mixture were then taken (0.4 ml in total, leaving 2 ml of the mix in the tube), 0.333 ml of xylene (C_8H_{10}, Merck) was added in the proportion 2.4 ml of mixture to 0.4 ml of xylene, the resultant mix being agitated for 60 s using a vortex mixer. Tubes were allowed to rest for 15 min at room temperature, permitting full separation of the two, aqueous and organic, phases. Thereafter, 0.4 ml of the aqueous phase were taken and the optical density was determined at 405 nm (OD_{405}; Bioscreen C MRB). The percentage of cells present in the solvent was calculated on the basis of an equation stating that the percentage of affinity to xylene was $100 \times [1 - (A/A_0)]$, where A_0 is the absorbance of the original suspension in saline solution prior to mixing with solvent, and A is the absorbance of the aqueous phase after mixing and resting. Each experiment was repeated three times with cultures prepared independently on three different days. Three categories of hydrophobicity were established on the following basis: weak if $< 21\%$, moderate if between 21% and 50%, and strong if $> 50\%$ (Capita et al., 2014).

3. Results and discussion

3.1. Minimum inhibitory concentrations (MICs) and adaptation

The MICs of the various different biocides for LM and SA are shown in Table 1. This table also shows the maximum concentrations of biocides permitting microbial growth after various passes through culture media at gradually increasing concentrations of the substances through adaptation.

TSP was the antimicrobial requiring the highest concentrations to inhibit growth of the two strains after 48 h of incubation, at 20,000 ppm for both microorganisms. Next came SHY, at 3500 ppm, equivalent to 350 ppm free chlorine, for LM, and 2500 ppm, equivalent to 250 ppm free chlorine, for SA. The values for MIC noted for SHY were similar to those observed in other studies performed on *L. monocytogenes* (Rodríguez-Melcón et al., 2018; 2019a). Similarly, they fell within the range recorded for other enterobacteria like *Yersinia enterocolitica* and *Cronobacter sakazakii* (Capita et al., 2019).

BZK was the biocide producing inhibition of growth at the lowest concentrations, 8 ppm for LM and 28 ppm for SA. These figures are similar to those seen previously in other research works. Values published include 2 ppm for Gram-positive bacteria such as MRSA, methicillin-resistant *Staphylococcus aureus* (Buzón-Durán et al., 2017), 3 ppm–13 ppm for *L. monocytogenes* (Rodríguez-Melcón et al., 2019b), 8 ppm for *S. enterica* serotype Typhimurium (Capita et al., 2017), 15 ppm for *C. sakazakii* and 20 ppm for *Y. enterocolitica* (Capita et al., 2019).

The MIC values recorded in the current study for PAA, at 1750 ppm for LM and 500 ppm for SA, are similar to those noted by Capita et al. (2019) for enterobacteria. However, they differ from other previous reports, such as that by Alonso-Hernando et al. (2009a), who found a MIC between 70 ppm and 80 ppm for *S. enterica* and between 100 ppm and 110 ppm for *L. monocytogenes*. These differences may be due to the fact that not all strains of a bacterium present the same susceptibility to biocides. Likewise, there may have been variations in the composition of the antimicrobial products used in the trials.

After several successive passes, each increasing one and a half times the concentration of the substance as bacterial presence grew, the maximum concentration of SHY permitting growth of the two microorganisms (2625 ppm for LM and 1875 ppm for SA) did not go beyond the MIC values. The conclusion was that these microorganisms had not undergone adaptation to this biocide. A similar state of affairs occurred with LM challenged with BZK and PAA, and with SA facing TSP, with no adaptation to these compounds being observed.

However, an adaptation was seen in LM treated with TSP, since after exposure the strain was able to grow in the presence of a concentration 2.53 times higher than the MIC for unexposed cultures. SA demonstrated adaptation to two biocides. With BZK, it tolerated a concentration of 31.5 ppm, in other words 1.13 times greater than the MIC for the unadapted strain, which stood at 28 ppm. With PAA, it achieved a tolerance 2.53 times greater, as the strain grew with a concentration of 1265.25 ppm after adaptation, whilst prior to exposure to the biocide the MIC had been 500 ppm. A similar reduction in susceptibility to biocides after repeated exposure of strains to sub-inhibitory concentrations was also observed in previous studies of *L. monocytogenes* (Alonso-Hernando et al., 2009a) and of enterobacteria, such as *E. coli* (Capita et al., 2014) or *S. enterica* (Alonso-Hernando et al., 2009a; Capita et al., 2017; Molina-González et al., 2014). Moreover, it has been demonstrated that exposure of bacteria to doses of biocides insufficient to kill them leads to cross-adaptation to a range of sub-lethal stress factors (Alonso-Hernando et al., 2009b). This reduction in susceptibility to biocides after their application at a weak concentration may be due to chromosomal mutations, triggering modifications in bacterial cells, principally in the composition and structure of their membranes, preventing penetration by antimicrobial agents. The resistance may also be associated with phenotypic modifications as a consequence of metabolic regulation of responses to stress induced by the presence of biocides, for example, increased expression of efflux pump mechanisms.

As indicated above, at times biocides are utilized at sub-inhibitory doses in food-processing environments (Capita et al., 2014; Virto et al., 2005). The results of the current research point to a need for extreme care in ensuring lethal concentrations of these substances are applied, so as to avoid rising tolerance of biocides, whether this relates to clinical contexts or to food industries (Capita et al., 2019).

Table 1
Minimum inhibitory concentrations (MICs) and maximum concentrations of biocides permitting microbial growth after adaptation for *L. monocytogenes* (LM) and *S.* Agona (SA).

Microorganism	Biocide	MIC (ppm)[a]	Maximum concentration permitting microbial growth (ppm)[b]
LM	SHY[c]	3500	2625
	BZK[d]	8	6
	PAA[e]	1750	1312.5
	TSP[f]	20,000	50,625
SA	SHY	2500	1875
	BZK	28	31.5
	PAA	500	1265.25
	TSP	20,000	15,000

[a] Minimum inhibitory concentration.
[b] Maximum biocide concentration permitting microbial growth after successive passes with gradually increasing concentrations of the substances.
[c] Sodium hypochlorite.
[d] Benzalkonium chloride.
[e] Peracetic acid.
[f] Trisodium phosphate.

3.2. Susceptibility to antibiotics

Infections caused by antibiotic-resistant bacteria are hard to treat, since many substances habitually used in clinical practice are ruled out as therapeutic options (Capita et al., 2019). Resistance to antibiotics is an increasingly major problem, nowadays considered one of the greatest challenges confronting public health around the world (Capita & Alonso-Calleja, 2013). This research determined whether exposure of

L. monocytogenes and *S. enterica* to low concentrations of biocides frequently employed in the food industry could contribute to a growing resistance to antibiotics. To this end, it checked susceptibility of the strains to ten antibiotics of clinical importance before and after exposure to increasing sub-inhibitory doses of the biocides tested. The results for MICs of these antibiotics for LM are shown in Table 2, and those for SA in Table 3.

Before exposure, LM showed resistance to all the antibiotics studied except erythromycin (E) and fosfomycin (FOS). Unlike the outcomes of the present research work, Alonso-Hernando et al. (2012) observed that all the strains of *L. monocytogenes* isolated from poultry in 2006 were susceptible to chloramphenicol and tetracycline. Nonetheless, those authors indicated that patterns of antibiotic resistance were changing over time, with resistant strains, and above all those with multiple resistance, increasingly common. This modification in patterns of susceptibility to antibiotics would appear to have arisen from the generalized employment of these substances, both in human and in veterinary medicine, since over-use or inappropriate utilization of antibiotics must be seen as the prime risk factor in the appearance of resistance among bacteria to these antimicrobials (Capita & Alonso-Calleja, 2013).

It is worth noting that erythromycin fortunately continues to demonstrate strong effects on LM. However, the resistance to ampicillin encountered is worrying, since this antibiotic, administered alone or in combination with gentamicin, is the treatment of choice against human listeriosis (Alonso-Hernando et al., 2012; Carvalho et al., 2019). In other studies, it has been noted that 20.9% (Jamali et al., 2015) or 67.6% (Carvalho et al., 2019) of strains of *L. monocytogenes* isolated from foodstuffs showed resistance to ampicillin.

After exposure to increasing sub-inhibitory concentrations of biocides, there was an enhancement relative to the unexposed strain in the resistance of *L. monocytogenes* to erythromycin (Table 2). PAA and BZK caused an increase in the strain's resistance to between ten (PAA) and forty (BZK) times MIC (0.10 ppm), as the microorganism achieved growth at concentrations of 1 ppm in the case of the first and of 4 ppm in that of the second. The most striking value was noted when the strain was exposed to TSP, since this permitted its resistance to erythromycin to be multiplied by 7500, with growth possible in the presence of 750 ppm of the antibiotic after adaptation. Studies undertaken by Capita et al. (2001a, 2001b) demonstrated that exposure of strains of *L. monocytogenes* to sub-inhibitory concentrations of TSP favoured the growth of this bacterium, suggesting that greater doses of the antibiotic would be needed to inhibit growth of the microorganism. For fosfomycin, a MIC of 30 ppm was recorded in the strain not exposed to biocides. However, the concentration of antibiotic which still allowed the strain to grow after exposure to the biocides was considerably higher, at 180 ppm for SHY, 240 ppm for BZK, 190 ppm for PAA, and 90 ppm for TSP. In the case of cefoxitin an increase in MIC was also observed after exposure to the biocides, although cultures had already been assigned to the resistant category in all instances. Something similar was observed by Alonso-Hernando et al. (2009b) with regard to strains of *L. monocytogenes* and *S. enterica*. Those authors discovered that susceptibility to antibiotics decreased in cells exposed to biocides, relative to those not so exposed, even if the values remained distant from the cut-off points established, so that they continued to be in the same susceptibility class.

In contrast, heightened susceptibility of LM to certain antibiotics was clear after challenge with increasing sub-inhibitory concentrations of biocides. One such case concerned chloramphenicol, with an MIC of 26 ppm in the strain not exposed to biocides, which would class it as resistant, whilst after exposure to biocides this strain was able to grow only with concentrations of chloramphenicol not exceeding a level between 6 ppm and 8 ppm. The MIC value for unexposed strains relative to ampicillin was 30 ppm, to vancomycin 38 ppm and to tetracycline 14 ppm. The maximum concentration of these antibiotics in which strains were able to grow after exposure to biocides dropped to 6 ppm in the strain challenged with SHY, 4 ppm in that exposed to BZK and 0.50 ppm in that exposed to PAA for the first of these antibiotics. The equivalent figures were 4 ppm for the strain challenged with PAA in the case of vancomycin and 7 ppm after exposure to this same biocide in respect of tetracycline. The maximum concentration of this antibiotic in which SA was able to grow after exposure to BZK dropped to 2 ppm. It should be pointed out that heightened susceptibility to certain antibiotics after exposure to sub-inhibitory concentrations of biocides is an interesting finding whose potential utility should be explored.

In respect of SA, before exposure this bacterium showed resistance to AMP, KF, E, C, and VA. Several studies indicate that enterobacteria produce alpha- and beta-lactamases, rendering them resistant to antibiotics like AMP (Bonardi et al., 2013; Fàbrega & Vila, 2012). The presence of efflux pumps in Gram-negative bacteria may also be linked to resistance to this antibiotic (Nikaido, 1996). Resistance to beta-lactams is a cause for great concern, since cephalosporins, such as cephalothin, are often utilized in treating salmonellosis in younger patients (Alonso-Hernando et al., 2009a).

After exposure to growing sub-inhibitory concentrations of biocides, *S. enterica* showed resistance or reduced susceptibility to several antibiotics to which it had been sensitive before exposure. This happened with cefoxitin (FOX, strain exposed to all biocides), gentamicin (CN, strain exposed to all biocides), fosfomycin (FOS; strains exposed to SHY, BZK and TSP) and enrofloxacin (ENR, strain exposed to BZK, PAA and TSP). For this last antibiotic a MIC of 0.06 ppm was recorded, so that it was classed as susceptible in accordance with the guidelines laid down by the CLSI (2018). Having been exposed to increasing sub-inhibitory concentrations of biocides, the strain was capable of growth in the presence of 0.10 ppm of the antibiotic when the challenge had been SHY, 0.75 ppm with BZK and 1.5 ppm with TSP (Table 3). However, the most striking result was noted after exposure of *S. enterica* to PAA,

Table 2
Minimum inhibitory concentration (MIC, ppm) of ten antibiotics against strains of *Listeria monocytogenes* with and without exposure to low doses of biocides.

Bacteria	Antibiotics (ppm)									
	AMP	KF	FOX	E	C	CN	TE	VA	FOS	ENR
LM non-exposed	30	190	40	0.10	26	15	14	38	30	>2300
LM exposed to SHY	6	550	440	0.05	8	6	60	130	180	>2300
LM exposed to BZK	4	650	1000	4	8	60	2	40	240	>2300
LM exposed to PAA	0.50	190	900	1	6	30	7	4	190	>2300
LM exposed to TSP	40	130	300	750	8	20	20	40	90	>2300
Threshold values	**1–1**	**0.12–0.5**	**4–8**	**1–1**	**8–8**	**1–1**	**1–2**	**2–2**	**32–32**	**0.5–4**

LM, *Listeria monocytogenes*. AMP (ampicillin), KF (cephalothin), FOX (cefoxitin), E (erythromycin), C (chloramphenicol), CN (gentamicin), TE (tetracycline), VA (vancomycin), FOS (fosfomycin), ENR (enrofloxacin). SHY, sodium hypochlorite; BZK, benzalkonium chloride; PAA, peracetic acid, TSP, trisodium phosphate. LM, *Listeria monocytogenes*. Threshold values were used to classify strains as susceptible (MIC ≤ lower threshold), of reduced susceptibility (MIC > lower threshold ≤ upper threshold), or resistant (MIC > upper threshold). These criteria in the case of AMP and E were specific to *L. monocytogenes* (EUCAST, 2021). Those for C, TE and FOS corresponded to *Staphylococcus* spp. and those for CN and VA to *Staphylococcus aureus* (EUCAST, 2021). Similarly, those for KF and FOX were likewise for *S. aureus* (CLSI, 2020) and those for ENR related to *Staphylococcus* spp. (CLSI, 2018).

Table 3
Minimum inhibitory concentration (MIC, ppm) of ten antibiotics against strains of *Salmonella* Agona with and without exposure to low doses of biocides.

Bacteria	Antibiotics									
	AMP	KF	FOX	E	C	CN	TE	VA	FOS	ENR
SA non-exposed	700	50	4	142	376	3	7	700	38	0.06
SA exposed to SHY	800	110	30	90	410	10	10	940	90	0.10
SA exposed to BZK	800	50	20	150	390	10	5	820	90	0.75
SA exposed to PAA	540	70	40	110	370	34	7	692	60	6
SA exposed to TSP	710	120	25	90	400	14	70	1000	100	1.50
Threshold values	**8–32**	**16–32**	**8–32**	**1–2**	**8–32**	**4–16**	**4–16**	**2–2**	**64–256**	**0.5–4**

SA, *Salmonella enterica* serotype Agona. The criteria for enterobacteria (CLSI, 2020) have been used. These criteria in the case of E and VA were specific to *Staphylococcus* spp. and *Staphylococcus aureus*, respectively (EUCAST, 2021). Those for ENR corresponded to *Staphylococcus* spp. (CLSI, 2018). For additional interpretation, see Table 2.

triggering a one-hundred-fold increase in resistance to ENR, as it was able to grow in the presence of 6 ppm of that antibiotic. Something similar was seen in the case of cefoxitin, which had a MIC for the unexposed strain of 4 ppm, so that its classification would be susceptible on the basis of CLSI guidelines. However, after exposure of *S. enterica* to the biocides under study, it grew in the presence of concentrations between 5 and 10 times higher than the MIC of the strain not exposed to biocides. Specifically, these concentrations were 30 ppm for SHY, 20 ppm for BZK, 40 ppm for PAA and 25 ppm for TSP. Similar results were recorded for gentamicin, where a MIC value of 3 ppm was observed for unexposed strain, while MICs of 10 ppm, 10 ppm, 34 ppm and 14 ppm were observed after exposure to SHY, BZK, PAA and TSP, respectively. With tetracycline, the MIC was 7 ppm for the unexposed strain, so it was classified as of reduced susceptibility. Heightened resistance to this antibiotic was recorded only in the strain exposed to TSP, which also achieved growth at 70 ppm of tetracycline.

Various authors have observed changes in patterns of susceptibility to a range of classes of antibiotics after exposure to biocides. This suggests that adaptation to biocides may be associated with broad-spectrum mechanisms like an enhanced expression of non-specific efflux pumps or modifications to the permeability of cell membranes (Capita et al., 2019; Condell et al., 2012; Randall et al., 2007).

3.3. Cell surface hydrophobicity

Several researchers have demonstrated that the hydrophobicity of the cell surface plays a critical part in bacterial adhesion to hydrophobic surfaces, like polystyrene, with later formation of biofilms (Gallardo--Moreno et al., 2002). Since adhesion of bacteria to a surface is the first step in the growth of biofilms, it is to be expected that increased hydrophobicity of the cell surface will be accompanied by a greater ability of bacteria to create biofilms on non-biotic surfaces of a hydrophobic nature (Norouzi et al., 2010; Patel et al., 2011; Takahashi et al., 2010).

Before exposure to sub-inhibitory concentrations of biocides, the percentage of affinity to xylene was lower ($P < 0.05$) in LM (23.09 ± 6.07%) than in that of SA (44.53 ± 5.57%), as shown in Figs. 1 and 2. After exposure to biocides, no differences ($P > 0.05$) were found between the percentage of affinity to xylene in LM exposed to SHY or to BZK and the control, with a moderate reaction being noted in all cases, with between 21% and 50% affinity to xylene. In contrast, the percentage of affinity to xylene in cultures of LM exposed to PAA (69.78 ± 10.44%; a strong reaction) and to TSP (49.81 ± 7.51%; a moderate reaction) was higher ($P < 0.05$) than the values noted under the remaining conditions.

With regard to the results obtained for SA strains exposed to biocides, no differences ($P > 0.05$) were noted between the percentage of affinity in those faced with SHY or with TSP and those not so challenged (control). In all cases moderate reactions of affinity for xylene were observed (from 21 to 50%).

SA confronted with PAA presented the lowest value for affinity to xylene (14.63 ± 3.95%), and that faced with BZK the highest (74.85 ± 7.51%). Previous studies (Alonso-Hernando et al., 2015) also observed that contact with some biocides could modify cell surface hydrophobicity upwards in Gram-negative microorganisms like *Escherichia coli*. Loughlin et al. (2002) indicated that Gram-negative bacteria, such as *Pseudomonas aeruginosa*, adapted to quaternary ammonium compounds (QACs) like benzalkonium chloride, presenting increased hydrophobicity of the cell surface when examined using a MATS test. This increase may be related to a reduction in negatively charged hydrophilic binding

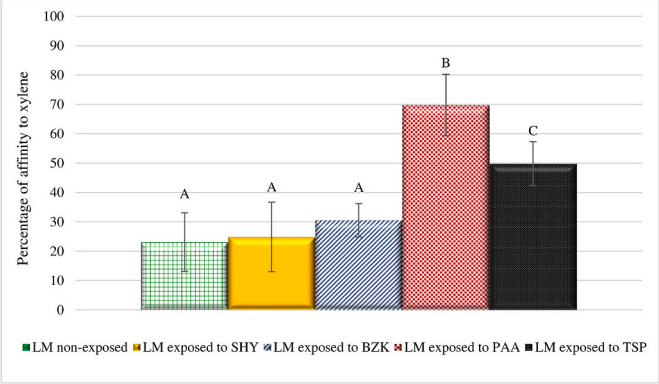

Fig. 1. Values for cell surface hydrophobicity in *L. monocytogenes* exposed to sub-inhibitory concentrations of biocides or non-exposed. LM, *Listeria monocytogenes*; SHY, sodium hypochlorite; BZK, benzalkonium chloride; PAA, peracetic acid; TSP, trisodium phosphate. Bars without letters in common are significantly different ($P > 0.05$).

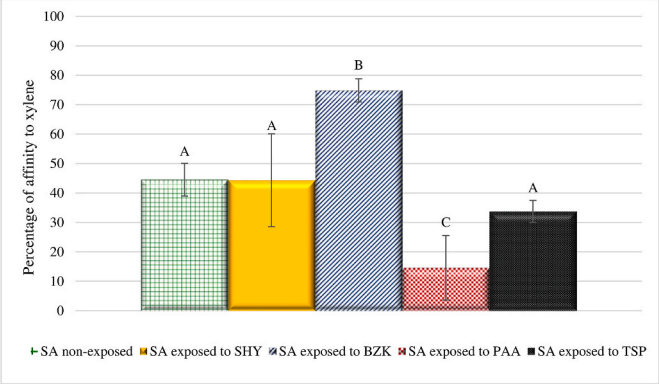

Fig. 2. Values for cell surface hydrophobicity in *S. enterica* exposed to sub-inhibitory concentrations of biocides or non-exposed. SA, *Salmonella enterica* serotype Agona; SHY, sodium hypochlorite; BZK, benzalkonium chloride; PAA, peracetic acid; TSP, trisodium phosphate. Bars without letters in common are significantly different ($P > 0.05$).

sites for the principal positive group in the biocide. As bacterial cells adapt to BZK, they tend to lose or mask binding sites for this compound. These sites are negatively charged and hence make a cell less hydrophobic, so that their being lost or masked increases cell hydrophobicity (Loughlin et al., 2002).

The higher hydrophobicity observed in both LM and SA after exposure to low doses of certain biocides is worrying, for two reasons. Firstly, hydrophobic materials are habitually used in food-processing environments (Rodríguez-Melcón et al., 2019a). Second, there is a link between heightened hydrophobicity and a greater capacity of strains de *L. monocytogenes* to form biofilms on this sort of surface (Rodríguez-Melcón et al., 2019c).

4. Conclusions

The effects of the different types and concentrations of biocides utilized in the food industry require in-depth investigation so as to optimize conditions of use. Since contact with low doses of certain biocides reduces susceptibility of *L. monocytogenes* and *S. enterica* strains to various antimicrobials, both biocides and antibiotics, as well as modifying cell surface hydrophobicity, which is linked to the capacity to form biofilms, stress must be laid on the essential need to apply biocides at a suitable concentration in food-processing contexts. The results from the present study may contribute to a better understanding of the behaviour of *L. monocytogenes* and *S. enterica* in the presence of weak concentrations of biocides. Further studies are required to determine genotype variations in these pathogenic microorganisms when exposed to sub-lethal concentrations of such compounds.

Credit author statement

Cristina Rodríguez-Melcón: Conceptualization, Validation, Formal analysis, Investigation, Writing - Original Draft. **Carlos Alonso-Calleja**: Conceptualization, Resources, Supervision, Project administration, Funding acquisition. **Rosa Capita**: Conceptualization, Methodology, Writing - Original Draft, Writing - Review & Editing, Supervision, Funding acquisition.

Declaration of competing interest

None.

Data availability

The authors are unable or have chosen not to specify which data has been used.

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