Listeria monocytogenes and *Salmonella enterica* Enteritidis Biofilms Susceptibility to Different Disinfectants and Stress-Response and Virulence Gene Expression of Surviving Cells

Diana Rodrigues,¹ Nuno Cerca,¹ Pilar Teixeira,¹ Rosário Oliveira,¹ Howard Ceri,² and Joana Azeredo¹

Disinfection of food contact surfaces is a challenging task, aggravated by bacteria's capacity to survive and/or resist antimicrobials by means of mechanisms not yet completely understood. This work evaluated the susceptibility of *Listeria monocytogenes* and *Salmonella enterica* biofilms to four disinfectants, and analyzed how those chemical agents influenced stress-response and virulence genes expression by surviving cells. Three strains of each bacterial species mentioned were used, and their biofilms were treated with sodium hypochlorite, benzalkonium chloride, hydrogen peroxide, and triclosan using the Calgary Biofilm Device. Expression of *L. monocytogenes* and *S. enterica* stress-response genes *cplC* and *ropS*, and virulence genes *prfA* and *avrA*, respectively, was analyzed through quantitative real-time polymerase chain reaction. Results showed sodium hypochlorite to have the lowest minimum biofilm eradication concentration values (3.125 µg/ml), whereas triclosan had the worst performance since no *S. enterica* biofilm eradication was achieved even at the maximum concentration used (4,000 µg/ml). *L. monocytogenes* stress-response gene and *S. enterica* virulence gene were significantly upregulated in surviving cells compared with controls. In general, this work points out sodium hypochlorite as the most effective disinfectant against biofilms of both species used, and *L. monocytogenes* biofilms to be more susceptible to disinfection than *S. enterica* biofilms. Moreover, it was found that disinfection surviving biofilm cells seem to develop a stress response and/or become more virulent, which may compromise food safety and potentiate public health risk.

Introduction

INADEQUATE CLEANING AND disinfection of food-processing environments is the cause of major economic losses and represents a serious danger to public health. In fact, several studies have shown that the presence of microorganisms on food contact surfaces is one of the most common causes of food spoilage and transmission of foodborne diseases,^{31,58,66} and their ability to adhere and form biofilms makes disinfection even more difficult and challenging.^{19,33,81} *Listeria monocytogenes* and *Salmonella enterica* are two of the most common foodborne pathogens responsible for numerous disease outbreaks worldwide every year,^{17,20,79} and numerous authors have reported that both these bacteria have the ability to adhere and form biofilms on many different surfaces.^{8,30,50} Moreover, the increased difficulty in eliminating adhered and biofilm forms of these microorganisms compared with planktonic cells has also been shown in several reports.^{15,22,37}

The bactericidal character of most commercial products used for surfaces cleaning and disinfection is mainly based on phenolic compounds, organic acids, alcohols, chlorine, quaternary ammonium compounds (QACs), and iodophors, the efficacy of which has been reported to be higher against bacterial suspensions than against adhered cells and biofilms.^{2,54,64} This fact has raised the need to reformulate the standard procedures used to test disinfectants' efficacy to include adhered cells and biofilms as targets together with planktonic cells.^{23,82,83} Among the various different methods that have been used to study biofilm communities,^{7,60,74} the Calgary Biofilm Device (CBD) is a high-throughput micro-titer plate-based technology for screening antimicrobial susceptibility of microbial biofilms.¹³ It was chosen to perform this work since it is a very versatile and high-throughput technique that allows minimum biofilm eradication concentration (MBEC) determination of a wide range of products and compounds such as antibiotics, biocides, metals, and disinfectants.^{3,27,29}

¹IBB—Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Braga, Portugal.
²Biofilm Research Group, Department of Biological Sciences, University of Calgary, Calgary, Canada.

Another important issue related to surfaces disinfection is the acquisition of bacterial resistance to disinfectant agents and, further, the possible relation between chemical biocides and the emergence of resistance to antibiotics. In fact, it has been thought that some biocides and antibiotics may have similar behaviors and characteristics in the way they act and in the way bacteria develop resistance to them.^{11,59,67,68} *L. monocytogenes* and *S. enterica* susceptibility and resistance to different kinds of antimicrobials has been widely studied, in both planktonic cells^{1,4,40} and biofilms.^{5,52,57} However, the effect of disinfection challenge on the expression of stress-response and virulence genes in these bacteria has not been so extensively studied, since only a few reports are available on this theme and all of them concern only planktonic cells.^{39,78,80} Moreover, to the authors' knowledge there is no report on genetic expression analysis of *L. monocytogenes* or *S. enterica* biofilm cells after disinfection challenge. Improving the knowledge about the relation between exposure to decontaminants and genetic responses would give further information for a more correct and cautious use of decontaminants in food-processing environments. In this context, the aims of the present work were to evaluate *L. monocytogenes* and *S. enterica* biofilms susceptibility to four commonly used disinfectants, and to investigate how their action may alter surviving cells' genetic expression concerning stress-response and virulence genes.

Materials and Methods

Bacterial strains and culture conditions

To assess the behavior of different strains from different sources, this work included three *L. monocytogenes* (food isolate 994, clinical isolate 1562, and reference strain CECT 4031^T) and three *S. enterica* Enteritidis strains (food isolate 355, clinical isolate CC, and reference strain NCTC 13349). All isolates were kindly provided by Dr. Paula Teixeira (Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Porto, Portugal). From a cryogenic stock at -70°C, strains were streaked out twice on trypticase soy agar (EMD Chemicals), and colonies were suspended in sterile saline (0.9% w/v) to match the optical density of a 0.5 McFarland standard. Suspensions were then diluted 1:30 in Mueller-Hinton II Broth cation adjusted (MH, Becton, Dickinson and Company) to a final concentration of $\sim 1.0 \times 10^7$ colony forming unit (CFU)/ml, which subsequently served as inocula for the assays. The starting cell number was always confirmed by plating three or four replicates of serial 10-fold dilutions of a sample of the inoculum.

Calgary biofilm device

The CBD was created in 1996 by microbiologists working at the University of Calgary and consists in a batch culture technique to grow 96 equivalent biofilms at a time.^{13,14} It is commercially available as the MBECTM physiology and genetics assay (Innovotech Inc.) and consists of 96 independent pegs mounted on the inside surface of the lid of a 96-well microtiter plate. Each peg fits the corresponding well when the CBD is placed over a microtiter plate, without contacting the well surface, allowing microorganisms to grow as 96 identical biofilms. By placing the biofilms on the pegs into the wells of a microtiter plate, it is possible to assess an array of antimicrobial compounds with varying concentrations.

Biofilm formation

Single strain biofilms were grown in CBD, the pegs of which were submerged in 200 μ l of inoculum placed in each well of the 96-well tissue culture plate. The device was placed on a gyratory shaker in a humidified incubator, where biofilms were left to grow at 37°C, for 24 hr at 125 rpm. After this incubation period, culture medium was discarded and biofilms on the pegs were washed for 1 min using 200 μ l saline 0.9% in each well of a microtiter plate. For biofilm growth control, eight individual pegs were broken off the MBEC peg lid using sterile forceps, placed into 200 μ l of recovery medium (MH + Tween 1%) and sonicated for 8 min on high with an Aquasonic (model 250HT; VWR Scientific)¹³ for biofilm disruption. Serial dilutions of the bacterial suspensions were made in 0.9% saline, plated on trypticase soy agar, and incubated for 24–48 hr at 37°C for subsequent CFUs count. Final data, given as log CFU/peg, resulted from at least three independent experiments with eight replicates each. All experimental conditions regarding biofilm formation were optimized to achieve a final biomass of about 6 log CFU/peg for all biofilms, to have countable amounts of cells even after a 3 log reduction caused by the disinfection assays.

Biofilms susceptibility tests

Disinfectants and neutralizer preparation. Four disinfectants were chosen for this study: (1) sodium hypochlorite (SH) solution, 4.99% wt/v available chlorine (Sigma-Aldrich); (2) PolycideTM, a commercial product in which the active agent is benzalkonium chloride (BAC) at 6.5% w/v (Pharmax Limited); (3) hydrogen peroxide (HP) 30% w/v solution in water (Sigma-Aldrich); and (4) triclosan (Sigma-Aldrich). Working solutions were prepared fresh at maximum concentrations of 800 μ g/ml for SH and BAC, 90 mg/ml for HP, and 4,000 μ g/ml for triclosan. These concentrations were first selected based on previous works that also studied the action of these chemical agents on bacterial cells,^{3,73} and were then adjusted if needed.

To inactivate disinfectants after biofilms challenge, it was used a universal neutralizer composed of reduced glutathione (Sigma Aldrich), L-histidine (Sigma Aldrich), and L-cysteine (Sigma Aldrich) dissolved in double-distilled water.^{12,28,76} For each disinfection challenge, a fresh solution of recovery medium plus neutralizer was prepared by adding 1 volume of universal neutralizer per 40 volumes of recovery medium, with final concentrations of 2.0 g/liter reduced glutathione, 1.0 g/liter L-histidine, and 1.0 g/liter L-cysteine. The effect of the neutralizer on bacteria viability was previously tested by submerging the biofilms in recovery medium supplemented with neutralizer. Biofilms were then sonicated and a sample of the suspension was plated out to compare the number of viable biofilm cells with the controls (viable cells from biofilms not subjected to the neutralizer action). No significant reduction in bacterial numbers was observed (data not shown).

Disinfection challenge. For disinfection assays, after identical biofilms were formed as described above, biofilms were washed for 1 min with 0.9% saline to remove free cells. The disinfection challenge was then performed by submerging the biofilms in the wells of 96-well tissue culture plates containing disinfectants solutions serially diluted (twofold) in phosphate-buffered saline for 15 min, at room

temperature, and without agitation. The pegs were then washed for 1 min with 0.9% saline to remove residual disinfectant solution and incubated for 1 min with the recovery medium + neutralizer (prepared as mentioned above) to inactivate the disinfectants. In the same plate, biofilms were sonicated for 8 min to promote disruption and recovery of surviving cells. Bacterial suspension dilutions and CFUs/peg counts were performed as described above.

Since for a disinfectant agent to be considered effective against adhered and biofilm cells it has to reach a 3 log units reduction,⁵¹ only the cells from biofilms that suffered such viability reduction were collected for later genetic expression analysis, as well as the corresponding biofilm cells that were not exposed to disinfection challenge (control). Moreover, for each bacterial species, only the most resistant strain to each disinfectant was selected for gene expression analysis. When different strains had the same MBEC value, the strain with the highest log CFU/peg value at the concentration immediately below MBEC was selected (data not shown). Collected cells were stored at -80°C in microtubes containing 500 μl of RNAlater[®] solution (Ambion).

Genetic expression analysis

Selected genes. To obtain information about how chemical disinfection may affect the genetic expression of biofilm cells, the following virulence and stress-response-related genes were selected for each bacterial species tested. Considering *L. monocytogenes*, *prfA* was the chosen virulence gene since it is the transcriptional activator of the main virulence genes of this species,^{16,21,41} with the known PrfA-regulated products, including surface proteins involved in host cell invasion and cell-to-cell spread and secreted membrane-damaging factors mediating escape from the phagocytic vacuole.^{18,55} To represent the stress-response-related genes of this bacterium, it was selected the *cplC* gene, as it encodes a protein (CplC ATPase) that is produced under stress conditions, both inside and outside the host, and that promotes early bacterial escape from the phagosome of macrophages enhancing intracellular surviving.⁶¹

Concerning *Salmonella* Enteritidis, virulence expression was assessed through *avrA* gene, a virulence-associated gene that is located within *Salmonella* pathogenicity island 1 (which is

necessary for the invasion of epithelial cells and induction of macrophage apoptosis), and that is involved in the induction of programmed cell death and the inflammatory response of hosts against infection.⁶ *rpoS* was chosen to represent the stress-response-related genes of this bacterium since it is the general stress-response regulator sigma factor required for surviving of bacteria under starvation and stress conditions.^{32,34,46}

Primer design. Primers used for *L. monocytogenes* and *S. enterica* stress-response and virulence genes analysis by quantitative real time-polymerase chain reaction (PCR) were designed using the software Primer 3,⁶² and are listed in Table 1. To verify the specificity of each primer pair for its corresponding target gene, PCR products were first amplified from genomic DNA (data not shown).

RNA extraction. Total RNA of each sample was extracted using the PureLink[™] RNA Mini Kit (Invitrogen) according to manufacturer's recommended protocol. Potential DNA contamination was removed during RNA purification procedure by On-column PureLink[™] DNase treatment (Invitrogen). RNA concentration (ng/ μl) and purity ($\text{OD}_{260\text{nm}}/\text{OD}_{280\text{nm}}$) were assessed by spectrophotometric measurement using a NanoDrop device (NanoDrop 1000 Spectrophotometer, V3.6.0, Thermo Fisher Scientific, Inc.).

cDNA synthesis. To ensure equivalent starting amounts of RNA from control and respective treated samples to be converted to cDNA, the proper dilutions in RNase-free water were performed. cDNA of each sample was synthesized using the iScript[™] cDNA Synthesis Kit (BioRad). Each reaction contained 2.5 μl of iScript Reaction Mix + iScript Reverse Transcriptase and 7.5 μl of RNA template, respecting the proportions recommended by the kit manufacturer in a final reaction volume of 10 μl . Complete reaction mix was incubated in a thermocycler (MyCycler[™] Thermal Cycler; BioRad) with the following reaction protocol: 5 min at 25°C , 30 min at 42°C , and 5 min at 85°C .

Quantitative real-time polymerase chain reaction. Real-time PCRs were performed on a CFX96[™] Real-Time PCR Detection System Bio-Rad system (Bio-Rad Laboratories,

TABLE 1. PRIMERS USED FOR THE ASSESSMENT OF GENE EXPRESSION BY QUANTITATIVE POLYMERASE CHAIN REACTION

Bacteria	Genes	Sequence (5'-3')	Product size (bp)
<i>Listeria monocytogenes</i>	<i>cplC</i>	F: CTTGGACCTACTGGTGTG R: TTGCCGAACTTTTCTGTC	197
	<i>prfA</i>	F: GGTAGCCTGTTTCGCTAATGA R: TAACCAATGGGATCCACAAG	193
	16S rRNA	F: GGAGCATGTGGTTTAATTCG R: CCAACTAAATGCTGGCAACT	199
<i>Salmonella enterica</i> Enteritidis	<i>ropS</i>	F: GAATCTGACGAACACGCTCA R: CCACGCAAGATGACGATATG	171
	<i>avrA</i>	F: GAGCTGCTTTGGTCCTCAAC R: AATGGAAGGCGTTGAATCTG	173
	16S rRNA	F: CAGAAGAAGCACCGGCTAAC R: GACTCAAGCCTGCCAGTTTC	167

Primers were designed using the software Primer 3.
bp, base pairs.

Inc.). Each 20 µl of reaction mixture contained 2 µl of cDNA (diluted 1:20 from the cDNA synthesis reaction), 1 µl of each primer, 10 µl of 2×SSoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Inc.), and 6 µl of nuclease-free water. Thermal cycling conditions were as follows: 3 min initial denaturation at 95°C, followed by 40 cycles of 10 sec denaturation at 95°C, 10 sec annealing at 50°C (for *L. monocytogenes* samples this step was performed at 53°C, concerning primers efficiency previously determined; data not shown), and a 15 sec extension at 72°C. A melt curve was performed at the end of each run, with readings from 65°C to 95°C every 1°C for 5 sec, to confirm that only the desired product was amplified.

Gene analysis and expression. Samples for real-time PCRs were run in triplicate. Data were analyzed using the Bio-Rad CFX Manager™ version 1.6 (Bio-Rad Laboratories, Inc.) and the relative quantification method ($2^{-\Delta\Delta C_T}$),⁴⁵ which describes the change in expression of the target genes relative to the 16S rRNA reference genes from untreated control samples.^{42,77} Data were analyzed by averaging the C_T values (cycle at which each sample amplification curve crosses a specific threshold) for triplicate samples. The ΔC_T values of the target genes were determined by normalizing to the endogenous control genes 16S rRNA. These samples were subsequently subtracted from the 16S rRNA genes from the untreated control samples. The $\Delta\Delta C_T$ was used to calculate relative expression using the formula $2^{-\Delta\Delta C_T}$.^{25,43,45} No-reverse transcriptase (no-RT) controls—RNA samples not submitted to the RT reaction—were used to check for possible DNA contamination. All no-RT controls showed $\Delta\Delta C_T$ values above 10 cycles, confirming the quality and purity of cDNA.

Statistical analysis

Quantitative PCR data were analyzed by means of the Student's *t*-test, at a 95% confidence level, using the statistical program SPSS (Statistical Package for the Social Sciences).

Results

Minimum biofilm eradication concentration

Results of biofilms susceptibility to each disinfectant presented in Table 2 revealed SH to have the lowest MBEC values for all biofilms tested, ranging from 3.125 to 12.5 µg/ml. On the other hand, the lower susceptibility was found in disinfection with triclosan, since it did not eradicate any of *S. enterica* biofilms even at the maximum concentration used (4,000 µg/ml). An intermediate susceptibility to BAC was found comparatively with the other compounds, with notably higher MBEC values than SH but considerably lower than those registered for HP and triclosan.

Intraspecies variability was found to influence the response to each chemical agent, with some strains being predominantly more resistant to disinfection, whereas others were more susceptible. In this way, *L. monocytogenes* clinical isolate 1562 and *S. enterica* clinical isolate CC were the most resistant strains to SH and HP, and SH and BAC, respectively. On the other hand, *L. monocytogenes* collection strain CECT 4031^T was the most susceptible to BAC, HP and triclosan actions, whereas among *S. enterica* strains only food isolate 355 revealed a lower MBEC value concerning disin-

TABLE 2. MINIMUM BIOFILM ERADICATION CONCENTRATION VALUES OF EACH DISINFECTANT AGENT

Strains	SH (µg/ml)	BAC (µg/ml)	HP (mg/ml)	Triclosan (µg/ml)
<i>L. monocytogenes</i>				
994	3.1	100.0	22.5	500.0
1562	6.3	50.0	45.0	500.0
CECT4031 ^T	3.1	6.3	11.3	250.0
<i>S. enterica</i> Enteritidis				
355	6.3	100.0	5.6	>4,000
CC	12.5	400.0	90.0	>4,000
NCTC13349	6.3	100.0	90.0	>4,000

SH, sodium hypochlorite; BAC, benzalkonium chloride; HP, hydrogen peroxide.

fection with HP (Table 2). Interspecies variability was also observed since, taking into account the average results of related strains in the same disinfection challenge, MBEC values against *L. monocytogenes* biofilms were inferior to those registered for *S. enterica*.

Stress-response and virulence gene expression

Results concerning gene expression by the most resistant *L. monocytogenes* and *S. enterica* strains to each disinfectant agent are presented in Fig. 1A, B and Fig. 1C, D, E, respectively. It was chosen to present these results graphically and per strain to enable an easier and faster observation of how each disinfectant has affected genetic expression. The first finding was that none of *L. monocytogenes* strains expressed the virulence gene *prfA* under any condition, neither before nor after disinfection (Fig. 1A-B), although its presence in genomic DNA was previously confirmed by PCR, as stated above. The same was also observed concerning expression of *rpoS* stress-response gene by *S. enterica* 355, before and after challenge with triclosan (Fig. 1C). In this way, only alterations of stress expression were registered for *L. monocytogenes* strains showing that, except for triclosan, all disinfectants lead to a significant increase of *cplC* gene expression by food isolate 994 and clinical isolate 1562. Stress expression by *S. enterica* strains was only notably altered in NCTC 13349 surviving cells after disinfection with HP (Fig. 1E), whereas CC biofilms treated with SH and BAC did not suffer significant alterations of *rpoS* gene expression (Fig. 1D). Except for SH, all disinfectants tested lead to a significant increase of virulence expression by *S. enterica* biofilm surviving cells, with triclosan promoting the highest increment on *avrA* expression, followed by HP and, finally, BAC.

The overall results showed HP to be the disinfecting agent with more effect on stress-response and virulence gene expression, followed by BAC, whereas SH had only affected stress expression by *L. monocytogenes* surviving cells. Triclosan was the only disinfectant that did not interfere with *cplC* gene expression, but, on the other hand, it was responsible for the highest *avrA* upregulation in *S. enterica* surviving cells.

Discussion

The present work evaluated *L. monocytogenes* and *S. enterica* biofilms' susceptibility to four commonly used disin-

FIG. 1. Relative expression of stress-response (gray) and virulence (black) genes of *Listeria monocytogenes* strains (A) 994 and (B) 1562, and *Salmonella enterica* strains (C) 355, (D) CC, and (E) NTCC 13349. Asterisks indicate significantly different values ($p < 0.05$) when comparing the relative expression of control (cont) and surviving biofilm cells. BAC, benzalkonium chloride; SH, sodium hypochlorite; HP, hydrogen peroxide.

fectant agents, and analyzed stress and virulence expression by the surviving cells. Biofilms from both bacterial species were more susceptible to SH than to any other disinfectant tested. Moreover, all SH MBEC values were way below the in-use recommended concentration (200 $\mu\text{g}/\text{ml}$), ranging between 3.13 and 12.5 $\mu\text{g}/\text{ml}$. This biocidal agent is a chlorine compound used as a disinfectant, the bactericidal effect of which is based on the penetration of the chemical and its oxidative action on essential enzymes in the cell.⁴⁷ On the other hand, *S. enterica* biofilms were resistant to triclosan, since this was the only disinfectant tested that did not achieve biofilm eradication. This compound is a bisphenol antimicrobial agent that has a broad range of activity,⁶³ being used as a preservative, antiseptic, and disinfectant in a diverse range of products.³⁶ In this study, a concentration range of 4,000–1.95 $\mu\text{g}/\text{ml}$ was used based on the fact that triclosan has been reported to be bacteriostatic at concentrations ranging between 0.025 and 100 $\mu\text{g}/\text{ml}$, and bactericidal at higher levels.^{26,71,72} Although MBEC values concerning *L. monocytogenes* biofilms ranged between 250 and 500 $\mu\text{g}/\text{ml}$, no *S. enterica* biofilms eradication was achieved by triclosan even at the maximum concentration used. This performance disparity concerning the two bacterial species used might be due to the fact that Gram-negative bacteria use multiple mechanisms to develop resistance to this antimicrobial agent.^{69,84} Moreover, it has been described

that the main physiological change resulting from adaptation to triclosan in *Salmonella* is the overexpression of efflux pumps.^{9,10} So, it is likely that these defensive mechanisms were taking place in *S. enterica* biofilm cells during disinfection and, thus, had prevented biofilm eradication.

Although not so susceptible as to SH, *L. monocytogenes* and *S. enterica* biofilms were also susceptible to BAC; most MBEC values were within the in-use recommended concentration for QACs, ~200 $\mu\text{g}/\text{ml}$. BAC is a nitrogen-based surface-active QAC with a broad-spectrum antimicrobial activity. Due to their positive charge, QACs form electrostatic bonds with negatively charged sites on bacterial cell walls, destabilizing the cell wall and cytoplasmic membrane, which leads to cell lysis, leakage, and death.^{49,70} Overall results obtained in this work showed a higher susceptibility of *L. monocytogenes* to BAC compared with *S. enterica* biofilms, although all *S. enterica* biofilms were also eradicated by this chemical agent, with only one case (CC strain) requiring a higher BAC concentration than that generally recommended.

Susceptibility tests performed with HP showed that some of its MBEC values were much higher than the 3% concentration that is generally present in disinfectants for surface wiping.³⁸ This chemical acts as a disinfectant by producing reactive oxygen species (hydroxyl radicals and superoxide anions), which attack essential cell components such as DNA, lipids, and proteins.³⁸ Although the effectiveness of

peroxides against biofilms has been recognized, previous reports have also shown that HP elicited a significant microbial reduction only at concentration ranges way above the target concentrations in the commercial mixtures.^{53,65,75}

Having determined the MBEC of each disinfectant tested, and identified the respective *L. monocytogenes* and *S. enterica* most resistant strains, expression of stress-response and virulence genes was analyzed. The first finding was that both control and surviving biofilm cells from the *L. monocytogenes* strains analyzed did not express the selected virulence gene (*prfA*) under the conditions studied; it was also possible that the expression was below the limit of detection of the assay. Although it is not clear what controls its activity or how *prfA* expression is regulated, it has been reported that the regulation of PrfA and virulence gene expression is influenced by several environmental factors. One example is the temperature-dependent control of translation of the *prfA* messenger, which is processed only at 37°C and not at 30°C.^{35,44} In the present work, disinfection challenges and collection of cells were performed at room temperature, which could be a reason why *prfA* expression was not detected. Moreover, intraspecies genetic expression variability is also another factor that may have caused this result, since it has been shown that genes with important functions can vary in their expression levels between strains grown under identical conditions.²⁴

On the other hand, disinfectants' actions lead to significant differences concerning the expression of stress-response genes by both bacterial species. As far as *L. monocytogenes* *cplC* gene is concerned, upregulations of almost threefold concerning SH and HP action, and twofold concerning BAC action were observed. In contrast, triclosan was the only disinfectant that did not interfere with *cplC* expression. As mentioned above, this gene encodes a protein that is produced under stress conditions and that promotes early bacterial escape from the phagosome of macrophages, enhancing intracellular surviving.⁶¹ So, SH, HP, and BAC actions upon *L. monocytogenes* biofilm cells may have triggered the same kind of stress conditions as those experienced by bacterial cells when inside a phagosome. In fact, one of the antimicrobial functions of phagocytic cells has been classified as an oxygen-dependent mechanism. Accordingly and as stated above, the mechanisms of action of SH and HP are mainly based on oxidative action, producing reactive oxygen species that attack essential cell components. In contrast, BAC acts mostly at the bacterial cells' wall and cytoplasmic membrane, destabilizing them and leading to death through cell lysis. A similar threat is presented to *L. monocytogenes* inside a phagosome, where lysozyme acts directly on the bacterial cell wall proteoglycans present especially in the exposed cell wall of Gram-positive bacteria.⁴⁸

Regarding the genetic analysis of *S. enterica* biofilms, the expression of the stress-response gene *rpoS* was only significantly increased after disinfection with HP. Besides being the general stress-response regulator sigma factor, *rpoS* has been reported to play an important role in biofilm formation,⁵⁶ which infers that its upregulation after treatment with HP may be a response to the damage caused by the free radicals produced by this chemical agent in the biofilm matrix.⁸³ Among *S. enterica* biofilms that were genetically analyzed, those formed by strain 355 were the only ones that did not express the *rpoS* gene. As stated above concerning *prfA*

gene expression, intraspecies gene expression variability is a likely reason of this occurrence.

Finally, the analysis of *avrA* gene expression by *S. enterica* biofilms showed that disinfection with triclosan, HP, and BAC leads to significant upregulations of about six-, five-, and twofold, respectively, compared with controls. However, SH was the only disinfectant that did not promote notable modifications on the expression of this gene. The substantial upregulation of this gene observed after treatment with triclosan is in agreement with a previous study that reported *S. typhimurium* biofilms response to this antimicrobial to include changes of gene expression.⁷³ In this way, our results not only corroborate these previous findings but also highlight that such bacterial response is not exclusively triggered by triclosan, since the same kind of genetic alteration was observed regarding *S. enterica* biofilms disinfection with HP and BAC.

Conclusions

The overall results of this work showed *L. monocytogenes* and *S. enterica* biofilms to be more susceptible to SH than to any other disinfectant tested, whereas all *S. enterica* biofilms were resistant to triclosan within the concentration range used. Save this case, all disinfection challenges were influenced by intra- and interspecies variability, as denoted by the different MBEC values observed after challenged with each disinfectant. Moreover, the overall results showed that the most resistant strains to each disinfection challenge had undergone genetic adjustments in terms of stress-response and/or virulence, depending on the bacterial species and strain. Consequently, the main finding of this work is the interesting and worrying fact that, even at concentrations that lead to significant reduction in biofilm biomass, disinfectants may induce virulence of the surviving cells and, thus, increase their infectious potential in case of contact with a host. Nevertheless, these studies are yet preliminary, since a higher number of strains need to be tested to address the high variability observed and, thus, reach more definite conclusions. Likewise, a wider range of target genes and disinfectants need to be studied to confirm the conclusions presented here, and to clarify which specific factors inherent to disinfection can be triggering the genetic changes of biofilm surviving cells.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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Address correspondence to:

Joana Azeredo, Ph.D.

IBB—Institute for Biotechnology and Bioengineering

Centre of Biological Engineering

University of Minho

Campus de Gualtar

4710-057 Braga

Portugal

E-mail: jazeredo@deb.uminho.pt