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Biofilm-forming ability and biocide susceptibility of *Listeria monocytogenes* strains isolated from the ready-to-eat meat-based food products food chain

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ABSTRACT

To assess the biofilm-forming ability of *Listeria monocytogenes* and its susceptibility to food-grade biocides, food (n = 120) and food contact equipment samples (n = 60) collected from the ready-to-eat meat-based food chain were analyzed. A total of 113 *L. monocytogenes* isolates were obtained and genetically characterized. Nineteen strains were tested for biofilm-forming ability and susceptibility to benzalkonium chloride, sodium hypochlorite and nisin. Most strains were moderate to strong biofilm-formers (crystal violet optical density ranging from 0.13 ± 0.03 to 0.20 ± 0.04). When treated with benzalkonium chloride and sodium hypochlorite, most biofilms were reduced, but the same did not happen with nisin. Three strains revealed a resistant profile to all biocides with high estimated LD_{90} values (≥1.79 mg/ml for benzalkonium chloride and sodium hypochloride and >1000 IU/ml for nisin). Biofilm-forming ability and LD_{90} values highlight the need to consider other sanitizers and novel strategies for mitigation and control of *L. monocytogenes* biofilms.

1. Introduction

Listeria monocytogenes is an opportunistic pathogen that causes severe foodborne disease in humans, with low incidence and high fatality rates (Auvolat & Besse, 2016). This bacterium is often associated with ready-to-eat meat-based food products (RTEMP) that are handled in operations such as cutting, slicing and packaging after listericidal treatment (Bolocan et al., 2016).

L. monocytogenes is believed to persist in food processing environments in biofilms, which exhibit a greater resistance to antimicrobials and environmental stresses than planktonic cells (Puga, SanJose, & Orgaz, 2016). *L. monocytogenes* mixed species biofilms are believed to be a major source of recontamination in RTEMP industries (Allen et al., 2016).

Chlorine-based disinfectants, such as sodium hypochlorite, are widely used in the food industry due to their broad-spectrum activity against bacteria, high efficacy and low cost (Waghmare & Annapure, 2015). Quaternary ammonium compounds, such as benzalkonium chloride, are cationic surfactants that are effective against various pathogens although developed resistance has been described (Ortiz, López, & Martínez-Suárez, 2014). Nisin, a *Lactococcus lactis* subsp. *lactis* bacteriocin, is a natural substitute for preservatives used in RTEMP. It has a generally recognized as safe (GRAS) status and presents bactericidal activity against *L. monocytogenes* (Fraqueza & Patarata, 2016; Jay, Loessner, & Golden, 2005).

Food-grade sanitizers are tested to prove effectiveness on the planktonic form of microorganisms, but the biofilm environment may change the response of every strain involved (Puga et al., 2016).

Previous work has been done assessing both the biofilm-forming ability and biocide resistance of *L. monocytogenes*, but to the best of the authors' knowledge, this is the first work that considers *L. monocytogenes* strains collected from the RTEMP food chain and assesses both their biofilm-forming ability and their susceptibility to biocides in use in those industrial and retail premises. Therefore, this study aimed to characterize the biofilm-forming ability of different *L. monocytogenes* strains collected in the RTEMP industry and retail, and evaluate their susceptibility to benzalkonium chloride, sodium hypochlorite and nisin.

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2. Materials and methods

2.1. Bacterial isolates collection and strains selection

A total of 120 final food products and 60 in-use and clean food contact surfaces were assessed for the presence of *L. monocytogenes*. Sampling occurred in the industrial and retail segments of the RTEMP food chain.

L. monocytogenes detection was performed according to ISO11290-1 and up to 10 presumptive colonies per sample were collected for PCR identification as described by Simon, Gray, and Cook (1996) and Talon et al. (2007). *L. monocytogenes* isolates (n = 113) were serogrouped by multiplex PCR (Kérouanton et al., 2010) and PFGE was performed according to PulseNet (Graves & Swaminathan, 2001).

For further testing, nineteen strains were selected to have representatives of different serogroups, pulsotypes and sampling points. These strains were preserved in brain heart infusion (BHI) broth (Scharlau Chemie S.A., Barcelona, Spain) with 15% glycerol at −80 °C and were revivified before use.

2.2. Biofilm production assay

The protocol proposed by Romanova, Gawande, Brovko, and Griffiths (2007) was used with some modifications to obtain a 5-day *L. monocytogenes* mono-cultural biofilm. Each strain was suspended in buffered peptone water (BPW) (Scharlau Chemie S.A.), incubated 16–18 h at 30 °C and afterwards the optical density at 600 nm (OD) was assessed (Pharmacia Biotech Ultrospec 2000, Virginia, Washington D.C.) to obtain a concentration of 8 log cfu/ml. Triplicate wells of a 96-well polystyrene flat-bottomed microtiter plate (Normax, Marinha Grande, Portugal) filled with 200 μl of BPW were inoculated with bacterial suspension to obtain a final concentration of 5 log cfu/ml, with three negative control wells containing BPW alone. The microtiter plates' OD was read in a SpectraMax 340 PC (Molecular devices, Silicon Valley, USA). The plates were covered, incubated 5 days at 30 °C and spent nutrients were removed daily and replaced with fresh BPW. By the end of the incubation period, the OD was measured. The control wells' average OD was subtracted from the test wells OD.

Crystal violet staining and viable cells enumeration in biofilm assays were performed in duplicate. *L. monocytogenes* CECT911 was used as a control due to its known adherence characteristics (Ibusquiza, Herrera, & Cabo, 2011).

2.2.1. Biofilm assessment by crystal violet staining

After removal of the medium, the microtiter wells were washed with sterile distilled water (SDW) to remove loosely associated bacteria, and air-dried for 45 min in the laminar flow hood. Each well was stained with 220 μl of 0.1% crystal violet (BioMerieux, France) solution for 15 min. After stain removal, the wells were washed three times with SDW and air-dried for 30 min in the laminar flow hood. Next, 220 μl of destaining solution (ethanol:acetone 80:20 v/v) was added to each well for 15 min. The microtiter plate was then shaken (Benchtop shaking incubator 222DS, Labnet International, Inc.) for 5 min and the crystal violet OD (cvOD) was measured in SpectraMax 340 PC. Each cvOD value was corrected by subtracting the average cvOD readings of negative control wells.

Adherence capability of the tested strains was based on the cvOD exhibited by bacterial biofilms, according to Stepanovic, Cirkovic, Ranin, and Svabic-Vlahovic (2004). The cut-off value ($cvOD_{c-o}$) was defined as 3 standard deviations above the negative control mean cvOD. The strains were classified as non-adherent ($cvOD \leq cvOD_{c-o}$), weakly adherent ($cvOD_{c-o} < cvOD \leq 2x\ cvOD_{c-o}$), moderately adherent ($2x\ cvOD_{c-o} < cvOD \leq 4x\ cvOD_{c-o}$) and strongly adherent ($4x\ cvOD_{c-o} \leq cvOD$).

2.2.2. Enumeration of viable cells in biofilms

After removal of the spent medium, the wells were rinsed with SDW to remove loosely associated bacteria, 100 μl of BPW was added to each well and a mini cell scraper (VWR International, Belgium) was used to detach biofilms from the well surface. The microtiter plate was sonicated (Ultrasonic bath MXB14, Grant Instruments, England) for 5 min to detach and collect sessile cells. Another 100 μl of BPW was pipetted into each well,10-fold dilutions were made in BPW and 10 μl were dropped on the surface of a tryptone soy agar (TSA) (Scharlau Chemie S.A.) plate. After overnight incubation at 30 °C, colonies were enumerated in a stereoscopic magnifier.

2.3. Biocide activity testing assay

Biocide activity testing in *L. monocytogenes* biofilms was performed according to European standard EN 1276:2009, using the quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants used in food and industrial areas, with the necessary adjustments for a microtiter plate.

Biocides were selected according to the ones that were being used to sanitize direct contact food surfaces and equipment in the sampled RTEMP industries (Henriques, Telo da Gama, & Fraqueza, 2014), i.e., sodium hypochlorite (HigiaBlue, Loures, Portugal) and benzalkonium chloride (Acros Organics, New Jersey, USA). Nisin (Sigma, St. Louis, USA) was also evaluated. Table 1 exhibits the tested concentrations for each biocide (diluted in hard water, according to EN 1276:2009) and respective neutralizers.

To simulate clean conditions, in all tests 0.03 g/l of bovine serum albumin (Sigma) was used as an interfering substance. Contact time (5 min) and temperature (20 °C) were established according to the obligatory test conditions specified in EN 1276:2009.

For all the isolates, experimental conditions were previously validated. Biocide activity was assessed using *Escherichia coli* DSMZ 682, *Pseudomonas aeruginosa* ATCC 15442, *Staphylococcus aureus* CECT 239, *Enterococcus hirae* ATCC 10541D-5, *L. monocytogenes* CECT 4031 (serogroup IIa), *L. monocytogenes* CECT 937 (serogroup IIb), *L. monocytogenes* CECT 911 (serogroup IIc), *L. monocytogenes* CECT 934 (serogroup IVa) and *L. monocytogenes* CECT 935 (serogroup IVb) strains. This previous assay was performed to validate experimental conditions (dilution-neutralization, absence of lethal effect in test conditions, including neutralizer toxicity) and efficacy of neutralizing solutions.

2.3.1. Biocide activity testing for L. monocytogenes 5-day old biofilms

To each well containing 5-day old biofilm, 20 μl of interfering substance and 20 μl of tryptone salt solution (Scharlau Chemie S.A.) were added. After 2 min, 160 μl of one of the diluted biocides containing 1.25x the desired test concentration was added, incubating for 5 min at 20 °C. After removal of the medium, the wells were washed with 40 μl of hard water and 160 μl of the appropriate neutralizer. After neutralization (5 min at 20 °C), the medium was removed and the wells were washed with SDW, which was also removed. Subsequent procedures were performed according to those described in 2.2.2. for biofilm detachment, dilution and colony enumeration. According to EN 1276:2009, cfu/ml was determined and log cfu/ml reduction (LogR) expressed as log10 reduction (log10 initial inocula - log10 final inocula) was calculated for each strain considering each biocide concentration. Whenever $LogR \geq 5$ is obtained, the biocide concentration is considered active.