Food Control 70 (2016) 371–379

Contents lists available at ScienceDirect

Food Control

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

Inactivation and induction of sublethal injury of *Listeria monocytogenes* in biofilm treated with various sanitizers

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ARTICLE INFO

Article history:
Received 3 May 2016
Received in revised form
14 June 2016
Accepted 14 June 2016
Available online 16 June 2016

Keywords:
Listeria monocytogenes
Biofilm
Sublethal injury
Sanitizer resistance

ABSTRACT

This study determined the effects of different sanitizers (one phenolic-based, one chlorine-based, two QACs-based and one levulinic acid and SDS-based) on *Listeria monocytogenes* biofilm. The induction of the sub-lethal injury state and the biofilm formation characteristics as a result of exposure to sanitizers were also evaluated. The results revealed that QACs-based and phenolic-based sanitizers most effectively reduced *L. monocytogenes*, resulting in a reduction of 3.7–6.9 log CFU/ml and 4.9–8.2 log CFU/ml after a 60-min treatment for 37°C- and 15°C-grown biofilms, respectively. An enhanced level of sanitizer resistance was observed in biofilms when they were multiply exposed to QACs-based and phenolic-based sanitizers, with a reduction of 0.7–3.5 log CFU/ml and 1.6–>9.3 log CFU/ml for 37°C- and 15°C-grown biofilms, respectively. As biofilm cells became less sensitive, especially to QACs-based sanitizers, an increase in the percentage of sublethally injured cells was observed to the levels dependent upon sanitizer concentration. Confocal laser scanning microscopy (CLSM) analysis revealed that biofilm cells experienced cell membrane damage when exposed to QACs-based and phenolic-based sanitizers, providing more protection to cells located inside the biofilm matrix. This study highlights the ongoing need for improvement in intervention methods to control *L. monocytogenes* in food processing plants.

1. Introduction

Bacterial biofilms in food processing environments are of recurrent concern to the food industry, mainly because of their strong antimicrobial tolerance (Kim, Hahn, Franklin, Stewart, & Yoon, 2009; Takenaka, Iwaku, & Hoshino, 2001). The limitation of agent penetration, multiple phenotypes development and existence of dormant cells, as well as the different reactivities of antimicrobial agents, are factors that influence biofilm tolerance to sanitizers (Kim, Pitts, Stewart, Camper, & Yoon, 2008). Furthermore, as biofilm forms, cells can detach and initiate attachment to other surfaces, providing potential transmission of spoilage- and disease-causing microorganisms (Yang et al., 2016). Despite improvements in plant layout, equipment design, and procedures for cleaning and sanitizing, the phenomenon of biofilms in the food industry is still poorly understood and controlled (Liu et al., 2015).

The presence of *Listeria monocytogenes* in food processing establishments is an important consideration. After entering the facility, *L. monocytogenes* can become a long-term resident, being able to persist for months or years in locations such as floor drains (Berrang, Meinersmann, Frank, Smith, & Genzlinger, 2005; Tompkin, 2002). The ability of *L. monocytogenes* to attach and form a biofilm e.g., on different processing plant surfaces, has been previously documented (Alonso, Perry, Regeimbal, Regan, & Higgins, 2014; Beresford, Andrew, & Shama, 2001; Gamble & Muriana, 2007; Zhao et al., 2013), and persistent subtypes are recognized as strongly adherent and especially capable biofilm producers (Berrang, Frank, & Meinersmann, 2010; Lundén, Miettinen, Autio, & Korkeala, 2000). Moreover, *L. monocytogenes* can attach to surfaces previously colonized by other bacteria and form mixed-species biofilms with, e.g., *Pseudomonas* (Hassan, Birt, & Frank, 2004). Importantly, *L. monocytogenes* cells have the potential to cross contaminate processing plant surfaces, even though a bactericidal treatment was applied (Reij & Den Aantrekker, 2004; Tompkin, 2002). The mechanisms by which cells survive under these conditions are not fully understood (Harvey, Keenan, & Gilmour, 2007). It is therefore believed that biofilms of *L. monocytogenes* play an important role in the survival of listeriae in the food processing environment (Tompkin, 2002; Zhao et al.,

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http://dx.doi.org/10.1016/j.foodcont.2016.06.015
0956-7135/

2013).

Cleaning and sanitizing procedures are widely used to inactivate and remove biofilms in the food industry (Yang et al., 2016). However, standardized methods for antimicrobial selection and for the design of effective strategy for biofilm control do not exist (Simões, Simões, & Vieira, 2009). Thus, in order to design an effective strategy, a better understanding of biofilm behaviour in response to various single and combined antimicrobials of different categories is essential. It is necessary not only to apply appropriate antimicrobials but also adjust the dosing, as bacterial cells may exhibit different sensitivity to a certain bactericidal concentration, depending on the mode of persistence (Kim et al., 2008; Simões et al., 2009). Accordingly, the development of adaptive response by biofilm cells as a result of bactericidal misuse should also be addressed with regard to the performance and maintenance of a strategy (Kim et al., 2008).

Because for the food industry, the control of *L. monocytogenes* in the food chain and the plant environment is of major concern, we examined *L. monocytogenes* LM101, serotype 4 isolated from food (salami isolate). Moreover, most listeriosis outbreaks have been caused by serotype 4b (Borucki & Call, 2003; Laksanalamai et al., 2014), hence *L. monocytogenes* LM101 is of the serotype most often associated with greater virulence; however further characterization is needed. In this study, the primary objective was to further elucidate the effects of different sanitizers on a *L. monocytogenes* biofilm. For this purpose, five sanitizers were selected and several parameters were tested, including temperature of biofilm growth, concentration of the sanitizers, time of exposure, and intervals of treatment application. Induction of the sublethal injury state and the biofilm formation characteristics as a result of exposure to sanitizers were also evaluated.

2. Materials and methods

2.1. Bacterial strain and preparation of single-species culture

Listeria monocytogenes strain LM101 (serotype 4, salami isolate) was used in this study. A culture was first grown at 37 °C in Tryptic soy broth (TSB, Becton Dickinson, Sparks, MD) for 24 h, and then subcultured into sterile TSB for another 5 h at 37 °C in order to reach mid-log phase. The single-species culture was prepared by diluting a mid-log phase culture in sterile TSB to a final concentration of ca. 2 log units of CFU/ml. The cell number was confirmed by plating on Tryptic soy agar (TSA, Becton Dickinson).

2.2. Biofilm growth and sanitizer treatment

The diluted mid-log phase culture of *L. monocytogenes* LM101 was added into 24-well flat-bottom polystyrene plates (Costar, Corning, NY) at 1 ml per well. The plates were incubated statically at 37 °C for 72 h and at 15 °C for additional 48 h to obtain ca. 8 log units of CFU. To maintain bacterial viability, the TSB was changed every 24 h by aspirating old medium from the walls of each well and dispensing fresh medium along the walls. After incubation, the wells were washed with 0.1 M phosphate buffer saline (PBS, Sigma, St. Louis, MO), and air-dried for 10 min. Two concentrations, in-use concentration (IUC) recommended by the manufacturer for use on hard non-porous surfaces and 1:1 in-use concentration (1:1 IUC), of commercially available surface cleaners and sanitizers: Vesphene® IIse (Steris, St. Louis, MO), FS Formula 12167™ (Zep, Atlanta, GA), Micronex® (Zep, Atlanta, GA), Fit-L Antibacterial Produce Cleaner – at the concentration typically used for washing produce, not sanitizing equipment (HealthPro Brands, Cincinnati, OH), and Zepamine A™ (Zep, Atlanta, GA) were used in this study. The composition and concentration of each sanitizer are listed in Table 1. All sanitizers were prepared with Milli-Q water (Millipore, USA) according to the manufacturers' instruction. Sterile PBS was used as a control. Sanitizers or PBS were removed by aspiration after 5, 15, 30 and 60-min treatment and then wells were filled with D/E neutralizing broth (Becton Dickinson), held for 10 min, and washed with sterile PBS. The content of each well was harvested by scraping the surface carefully with a sterile polyester-tipped swab (15.2 cm; Fisher Scientific). The swabs were placed into 9-mL PBS tubes, and vigorously (250 rpm) agitated by a Vortex mixer (G-560, Scientific Industries, Bohenia, NY) for 30 s. Cell suspension (100 μL) or appropriate dilutions in 0.01 M PBS were spread plated in duplicate onto TSA plates. The colonies on the plates were counted after 24 h of incubation at 37 °C. The level of inactivation was expressed as the $\log_{10}$ reduction in the cell survival ratio for the sanitizer treatments ($\log N/N_0$). N refers to the bacterial counts after sanitizer treatments, whereas N_0 refers to the bacterial counts following PBS incubation (control biofilms). Sublethal injury was determined by simultaneously spread plating the treated cells on TSA supplemented with sodium chloride (NaCl; Fisher Scientific, Pittsburgh, PA) plates. In a preliminary study, several concentrations of NaCl (1–6% w/v) were tested to determine the maximum concentration that did not affect the growth of healthy, untreated cells (Ghate et al., 2013) (data not shown). Based on the results obtained, 3% NaCl was selected for use in the sublethal injury test. The following formula was used to calculate the percentage of sublethal injured cells:

$$\textit{Sublethal injury}\ (\%) = \left(1 - \frac{\text{Colonies on TSA} + \text{NaCl}}{\text{Colonies on TSA}}\right) \text{x } 100$$

The second treatment using the same exposure times was conducted after a 24-h biofilm recovery in TSB at 37 °C or 15 °C for all sanitizers. Biofilm cell inactivation and cell injury after the 2nd treatment were determined by enumeration and the sublethal injury test as described above. The most effective sanitizer treatments were repeated at one-week intervals during a three-week biofilm incubation at 37 °C or 15 °C by exposing biofilms for 60 min to sanitizers, and then the same enumeration procedure and sublethal injury test were performed.

2.3. Confocal laser scanning microscopy (CLSM)

For CLSM, the inoculum of *L. monocytogenes* LM101 was prepared as described in Section 2.1 and added to 8-well chamber slides (Nunc™ Lab-Tek™, Fisher Scientific) at 400 μL per well. The plates were incubated at 37 °C for 72 h or at 15 °C for an additional 48 h before sanitizer treatments. The medium was changed every 24 h to maintain bacterial viability. Prior the treatment step, the wells were washed with 0.1 M PBS and air-dried for 10 min, and after treatments were filled with N/E neutralizing broth to neutralize the residual sanitizer solutions. To visualize cells, fluorescent dyes were used by applying the LIVE/DEAD BacLight™ viability kit (Molecular Probes, LifeTechnologies, Eugene, OR). The staining mixture was prepared by adding 3 μL of component A and 3 μL of component B per 1 mL of sterile saline. Freshly prepared staining mixture (400 μL) was added to each well and incubated at 21 °C for 15 min in the dark. After incubation, the wells were washed with PBS as described above. The wells were further filled with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (Sigma) and incubated for 30 min at room temperature to fix the specimen. The slides were removed from the chambers and then covered with coverslips with the use of BacLight™ Mounting Oil (Molecular Probes, LifeTechnologies, Eugene, OR). The samples were analyzed with a Zeiss LSM 700 CLSM (Carl Zeiss Microscopy, Thornwood, NY) using Plan-Apochromat 63 × oil-immersion, numerical aperture 1.4