Food Control 65 (2016) 143–151

Contents lists available at ScienceDirect

Food Control

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

Biofilm development at low temperatures enhances *Listeria monocytogenes* resistance to chitosan

C.H. Puga, C. SanJose, B. Orgaz*

Department of Nutrition, Food Science and Technology, Faculty of Veterinary, University Complutense of Madrid (UCM), Avda Complutense s/n, 28040 Madrid, Spain

ARTICLE INFO

Article history:
Received 7 October 2015
Received in revised form
7 January 2016
Accepted 8 January 2016
Available online 11 January 2016

Keywords:
Listeria monocytogenes
Dual-biofilms
Cold stress
Chitosan
CLSM
Pseudomonas fluorescens
Revitalization

ABSTRACT

The persistence of certain *Listeria monocytogenes* (*Lm*) strains in the food industry is a concerning food safety issue. Most of the studies that link persistence with other features, such as biofilm (BF) formation and tolerance to antimicrobials, are conducted at different temperatures from those generally used in food facilities. In this work, two *Lm* strains, one persistent and one sporadic, plus the reference strain Scott A were cultivated either alone or with *Pseudomonas fluorescens* (*Pf*) to form BF at 20 °C or 4 °C. In both systems, the ability of *Lm* cells to recover after chitosan treatment was evaluated. Structural changes after chitosan exposure and over the revitalization period were analysed using confocal laser scanning microscopy (CLSM). More *Lm* cells per surface unit were attained in binary BF formed at room temperature, though in those formed under refrigeration, higher tolerance to chitosan was observed. Whereas the persistent *Lm* strain recovered from chitosan damage at almost the same rate with or without *Pf*, the presence of this accompanying species was crucial for non-persistent *Lm* strain recovery, especially at those BF formed at 4 °C. The temperature at which BF were formed, influenced the morphology of the lesions caused by chitosan, as well as the time profile of *Lm*'s viability recovery, suggesting that cold stress increases the tolerance to this antimicrobial, either through structural BF modification, or in some other way. These findings are relevant to the design of realistic BF control strategies in food industry, where most products are handled under refrigerated conditions.

1. Introduction

Listeria monocytogenes (*Lm*) can persist in food processing facilities for extended periods of time (Keto-Timonen, Tolvanen, Lundén, & Korkeala, 2007; Ortiz et al., 2010) and can eventually be transferred from food contact surfaces to food. Although its incidence is relatively low compared to other zoonotic agents (the EU notification rate was 0.44 cases per 100,000 population), the EU case-fatality rate due to listeriosis was the highest one for illnesses caused by foodborne pathogens, at 15.6% (191 deaths reported in the EU in 2013) (EFSA, 2015). The features by which certain *Lm* strains persist in food environments are not fully understood to date. Nevertheless, it seems that association with other microorganisms, most likely forming multispecies biofilms (BF) is remarkably meaningful for their susceptibility to cleaning and disinfection procedures (Carpentier & Cerf, 2011; Jahid & Ha, 2014; Srey, Jahid, & Ha, 2013; Valderrama & Cutter, 2013).

Biofilms are microbial communities that grow attached to surfaces and are embedded in an extracellular matrix (ECM) produced by themselves (Donlan & Costerton, 2002). In real environments, BF are frequently formed by different species that inhabit the same niche (Elias & Banin, 2012). In general, interspecies interactions may change biocide tolerance response of every strain involved (Giaouris et al., 2015; Simões, Simões, & Vieira, 2009). The effect of *Lm* associations in mixed BF has been described as positive, neutral or even detrimental, depending on the partners, the antimicrobials used and the culture conditions (Giaouris, Chorianopoulos, Doulgeraki, & Nychas, 2013; Kostaki, Chorianopoulos, Braxou, Nychas, & Giaouris, 2012; Lourenço, Machado, & Brito, 2011; Van der Veen & Abee, 2011).

Environmental conditions in the food industry context may include different stress situations that certain strains of *Lm* can adjust to, such as desiccation, starvation, low pH and low temperature (Bergholz, Bowen, Wiedmann, & Boor, 2012; Chaturongakul, Raengpradub, Wiedmann, & Boor, 2008; Valderrama & Cutter, 2013). In food processing or handling premises, most of the zones

* Corresponding author.
E-mail address: belen@vet.ucm.es (B. Orgaz).

http://dx.doi.org/10.1016/j.foodcont.2016.01.012
0956-7135/

are maintained under refrigeration temperatures (usually lower than 5 °C), especially those of cleanrooms where Ready to Eat (RTE) foods are dealt with. When testing antimicrobials intended for the food industry use, this fact is often disregarded and target BF are cultivated at relatively high temperatures. Some evidence however suggests that persistence of certain strains may be enhanced by low temperature adaptation mechanisms (Cabrita, Trigo, Ferreira, & Brito, 2015; Lourenço et al., 2011; Ochiai et al., 2014). For food safety reasons, it is therefore relevant to find out whether cultivation at low temperatures and presence of accompanying microorganisms alter *Lm's* tolerance to antimicrobials. *Pseudomonas* species are psychrotrophic spoilage organisms very commonly found in food related settings. Thus, *Lm/Pseudomonas fluorescens* BF were developed at 4 °C and 20 °C. Two of the *Lm* strains used here, one persistent and the other non-persistent, were isolated from an Iberian pig slaughterhouse by Ortiz et al., (2010); reference strain Scott A was used for comparison. These dual-species BF were treated with chitosan, a natural biopolymer with demonstrated antiBF effectiveness (Costa, Silva, Tavaria, & Pintado, 2013; Martinez et al., 2010; Orgaz, Lobete, Puga, & SanJose, 2011; Orgaz, Puga, Martínez-Suárez, & SanJose, 2013). Moreover, to determine whether the ability of *Lm* to recover from chitosan action is affected by the presence of *Pf* and temperature, the *Recovery Rate* of *Lm* cell viability along a 48 h revitalization period, was measured. Changes in live/dead subpopulations and BF structure were also observed over this period by confocal laser scanning microscopy (CLSM).

2. Materials and methods

2.1. Bacterial strains

P. fluorescens ATCC 948™ (*Pf*) and three *L. monocytogenes* (*Lm*) strains, the reference strain *Lm* Scott A (serotype 4b, lineage I), and two environmental strains, *Lm* 10p and *Lm* 6np isolated from an Iberian pig slaughterhouse and processing plant by Ortiz et al. (2010) were used as BF forming microorganisms. *Lm* environmental isolates were identified and characterized by serotyping and PFGE (Pulsed Field Gel Electrophoresis) by the same authors. *Lm* 10p was one of the strains classified as persistent as it had been repeatedly sampled over a large time span (from 1 to 3 years), while 6np was in the non-persistent group. They were all stored at −20 °C in Tryptone Soya Broth (TSB) (Oxoid) with 15% glycerol. Preinocula cultures were incubated overnight under shaking (80 rpm) at 20 °C in TSB to attain the mid exponential phase. Cells harvested by centrifugation at 4000 × g for 10 min, washed twice in sterile TSB and with adjusted OD_{600} (0.12) were inoculated to start with 10^4 CFU mL^{-1} both monospecies and dual-species cultures.

2.2. Experimental system

BF were developed on 22 × 22 mm thin, commercial microscope borosilicate glass coverslips, as described by Orgaz et al., (2011). These coverslips provide single-use, cheap, clean and undamaged smooth surfaces, without scratches or other microtopographic irregularities, moderately more hydrophilic than stainless steel, but allowing for more reproducible BF than reusable metal coupons. Sixteen coverslips were held vertically by marginal insertion into the narrow radial slits of a Teflon carousel platform (6.6 cm diameter). The platform and its lid were assembled by an axial metallic rod for handling and placed into a 600 mL beaker (Fig. 1). The whole system, *i.e.*, coverslips, carousel and the covered 600 mL beaker were heat-sterilized as a unit before aseptically introducing 60 mL of inoculated TSB. For dual-species BF containing *Pf* and one of the three *Lm* strains, both bacterial species were inoculated to obtain the same initial level of 10^4 CFU mL^{-1}. *Lm* monospecies BF were used as controls. Incubation was carried out at either 20 °C for 48 h (*warm* BF) or 4 °C for 20 days (*cold* BF) in a rotating shaker at 80 rpm. Under these conditions, BF growth covered approximately 70% of the coverslip's surface.

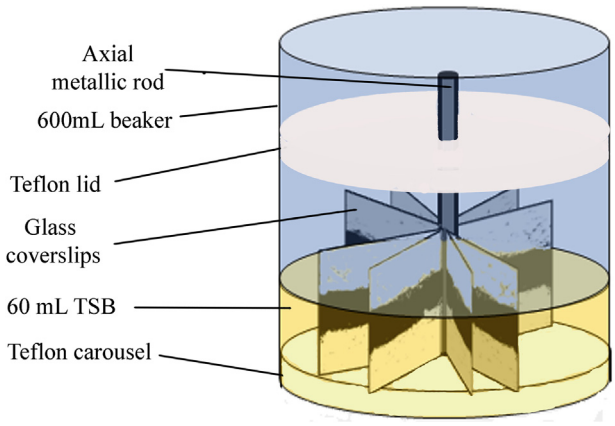

Fig. 1. Experimental system. Carousel for biofilm development.

2.3. Chitosan tests

Chitosan with a degree of deacetylation of 75–85% and MW ranging from 190,000 to 300,000 Da was purchased from Sigma Aldrich. For BF assays, a 1% (w/v) chitosan solution was prepared in 1% (v/v) acetic acid. For chitosan exposure, the coverslips carrying BF were aseptically removed from the carousel platform with sterile tweezers and dipped into sterile NaCl (0.9% w/v) to eliminate weakly attached cells. Next, the samples were individually immersed into Falcon test tubes containing 15 mL of the sterile chitosan cleaning solution for 1 h at 20 °C followed by a washing step with saline before viable cell retrieval and quantification.

2.4. Cell retrieval and counting

For viable cell retrieval and counting, attached cells from control or treated coverslips were removed by swabbing both sides. Cells transferred into test tubes with 1.5 mL of peptone water, were vigorously mixed in a vortex stirrer to break up cell aggregates, decimally diluted in peptone water and pour-plated. *Pf* and *Lm* counts were quantified in selective media (PALCAM for *Lm* and Pseudomonas selective agar for *Pf*, all from Oxoid) counting after 48 h incubation at 37 °C. For purity control, plating on Tryptone Soya Agar (TSA, also from Oxoid) was used to spot visually different colonies. Chitosan antimicrobial efficiency was expressed as log reduction of attached cells. All cultures were run in triplicate on different days and two coverslips were taken from a carousel for each treatment. Data thus correspond to an average of six measurements.

2.5. Biofilm cell recovery after chitosan exposure

To monitor the recovery of viable attached cells exposed to chitosan, treated *warm* and *cold* BF individual coverslips, were aseptically placed into sterile Falcon test tubes containing 15 mL of fresh sterile TSB and were incubated at 20 °C while shaking. Sampling for BF viable cell quantification was carried out after 5, 16, 24 and 48 h. *Recovery Rates* were defined as the reverse of the *apparent* generation times (1/g) of the attached counts during the revitalization period. *Apparent* here means a combination of overall