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Biofilm-producing ability of *Listeria monocytogenes* isolates from Brazilian cheese processing plants

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ABSTRACT

The persistence of *Listeria monocytogenes* in food industry environments has been associated to the ability of specific isolates to produce biofilms. This study aimed to evaluate the biofilm production of 85 *L. monocytogenes* strains previously isolated from samples of cheese, brine and the environment of two cheese processing plants located in São Paulo, Brazil. The *L. monocytogenes* isolates belonged to serotypes 4b, 1/2b and 1/2c, yielded 30 different pulsotypes by pulsed-field gel electrophoresis (PFGE), and were submitted to biofilm-formation assays on polystyrene microplates and stainless steel coupons incubated statically at 35 ± 0.5 °C for 48 h. All isolates from different sources showed ability to produce biofilms on polystyrene microplates, from which 21 (24.7%) also produced biofilms on stainless steel. Four isolates (4.7%) belonging to four different pulsotypes were classified as strong biofilms-producers on polystyrene microplates, while isolates belonging to four pulsotypes previously evaluated as persistent had weak or moderate ability to produce biofilms on polystyrene microplates. No relationship between the serotypes or pulsotypes and their biofilm-forming ability was observed. This study highlights the high variability in the biofilm production among *L. monocytogenes* strains collected from cheese and cheese-production environment, also indicating that strong biofilm-formation ability is not a key factor for persistence of specific isolates in cheese processing plants.

1. Introduction

Listeria monocytogenes is a human pathogenic bacterium which is able to survive in several food processing environments in wide range of temperature, pH, salt concentration, and at low water activity (Gandhi & Chikindas, 2007). In dairy plants, *L. monocytogenes* can persist for long period of time in multiple places such as drains, walls, ceilings, storage tanks, hand trucks, conveyor belts and other sites that can accumulate food residues (Rückerl et al., 2014; Todd & Notermans, 2011). Fresh cheeses usually present pH-values ≥4.4 and a_w ≥0.94, which may favor the growth of foodborne pathogens (Ryser, 2011). Moreover, fresh cheeses have been frequently linked to human listeriosis outbreaks in several countries (Jackson et al., 2011; Melo, Andrew, & Faleiro, 2015), and are considered high-risk foods for *L. monocytogenes* contamination.

Several studies indicate that *L. monocytogenes* isolates are able to adhere and produce biofilms on inert surfaces (Shi & Zhu, 2009; Takahashi et al., 2011), hence forming an organized and protected community of cells (Jung, Choi, Kim, Lee, & Kwon, 2015). The development of bacterial biofilms on surfaces is mainly favored by inadequate cleaning and disinfection procedures, especially in areas with difficult access, such as tubes, valves and heat exchangers in dairy industries (Bridier et al., 2015). Moreover, the protective effect provided by the biofilm structure may enhance the resistance of bacterial cells to sanitizing agents (Belessi, Gounadaki, Psomas, & Skandamis, 2011), creating a permanent source of contamination in the food processing facilities. The persistence of *L. monocytogenes* in food industry environments has been associated with the ability of specific isolates to produce biofilms (Aase, Sundheim, Langsrud, & Rørvik, 2000; Borucki, Peppin, White, Loge, & Call, 2003). However, contradictory reports indicated that persistent *L. monocytogenes* isolates were not stronger biofilm producers, compared to non-persistent ones (Harvey, Keenan, & Gilmour, 2007; Nilsson, Ross, & Bowman, 2011).

L. monocytogenes has been frequently isolated from dairy products worldwide (Brito et al., 2008; Ismaiel, Ali, & Enan, 2014; Kevenk & Terzi Gulel, 2016; Schoder, Stessl, Szakmary-Brändle, Rossmanith, & Wagner, 2014), with some investigations indicating persistence of

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lineages in the environment of cheese processing plants in Brazil (Barancelli et al., 2014). However, the biofilm-producing ability of isolates from Brazilian dairy industries has not been investigated. Thus, the present study aimed to evaluate *L. monocytogenes* isolates obtained from cheeses, brines and environment surfaces of two cheese processing plants located in the state of São Paulo, Brazil, for their ability to adhere and produce biofilms on polystyrene microplates and stainless steel coupons.

2. Materials and methods

2.1. L. monocytogenes isolates and pulsotypes

Eighty-five *L. monocytogenes* isolates were evaluated in this study, which were previously obtained from 257 samples collected in two cheese processing plants located in the northeastern region of the state of São Paulo from October 2008 to September 2009, as described by Barancelli et al. (2014). The isolates were obtained from samples of drain in cooling chamber ($n = 16$), floor of pasteurization room ($n = 8$), floor of cooling chamber ($n = 32$), plastic crates ($n = 8$), platform of cooling chamber ($n = 7$), surfaces of worker's gloves ($n = 3$), brine ($n = 5$) and cheese ($n = 6$). The 85 *L. monocytogenes* isolates belonged to serotypes 1/2b, 1/2c and 4b, and yielded 30 different pulsotypes by pulsed field gel electrophoresis (PFGE), as presented in Table 1. Four pulsotypes (XIV, XVI, XVII and XVIII) were considered persistent in the cheese plants evaluated since they were isolated at least 2 different sampling times (Barancelli et al., 2014). The isolates were maintained in tubes containing brain heart infusion (BHI; Oxoid, UK) broth with 15% glycerol at −80 °C until analyses for biofilm formation ability.

Table 1
Listeria monocytogenes isolates evaluated in this study, previously sero- and molecular typed by Barancelli et al. (2014).

Pulsotype[a]	Number of isolates	Persistent[b]	Serotype	Cheese plant origin
I	4	No	1/2b	A
II	2	No	1/2b	A
III	1	No	1/2b	A
IV	1	No	1/2b	A
V	1	No	1/2b	A
VI	1	No	4b	B
VII	1	No	4b	B
VIII	2	No	4b	A
IX	2	No	4b	A
X	1	No	4b	A
XI	1	No	4b	B
XII	1	No	4b	B
XIII	1	No	4b	B
XIV	3	Yes	4b	A, B
XV	2	No	4b	A
XVI	2	Yes	1/2b	B
XVII	6	Yes	4b	A, B
XVIII	31	Yes	4b	A, B
XIX	1	No	4b	A
XX	1	No	4b	A
XXI	4	No	4b	B
XXII	3	No	4b	A
XXIII	1	No	4b	A
XXIV	1	No	1/2b	A
XXV	2	No	4b	B
XXVI	3	No	4b	B
XXVII	2	No	4b	B
XXVIII	1	No	4b	B
XXIX	1	No	4b	B
XXX	2	No	1/2c	B

[a] $N = 30$ pulsotypes, as defined by pulsed-field gel electrophoresis (PFGE).
[b] Persistence accepted when the pulsotype was isolated at least 2 different sampling times from Nov/2008 to Sep/2009.

2.2. Biofilm formation assays on polystyrene microplate

Biofilm assays were conducted on polystyrene microplates by using the methodology described by Stepanović, Vuković, Dakić, Savić, and Švabić-Vlahović (2000) with some modifications, as follows. One loop of each *L. monocytogenes* isolate was added to 5 mL of Tryptone Soya Broth (TSB; Oxoid, UK) and incubated at 37 ± 0.5 °C for 24 h. After this period, the culture broths were diluted until reaching approximately 10^8 cells mL^{-1} using a McFarland scale. Triplicate aliquots (200 μL) of each TSB bacterial suspension were transferred into 3 wells of flat bottomed, 96-well polystyrene microplate and incubated statically at 35 ± 0.5 °C, to mimicking the typical average temperatures (>30 °C) in cheese processing environments in Brazil, for 48 h (Djordjevic, Wiedmann, & Mclandsborough, 2002). After incubation, microplates were washed with sterile phosphate-buffered saline (PBS, pH 7.2), fixed with methanol (Synth, Brazil), stained with crystal violet 0.1% (Synth, Brazil) for 15 min, dried, and resolubilized with 33% (v/v) glacial acetic acid (Synth, Brazil). The optical density (OD) of each well was measured at 570 nm using a microtiter plate reader (LabSystems, MultiSkan, USA). Triplicate negative controls (NC) with only sterile TSB were used as reference for determination of the ability of *L. monocytogenes* isolates to produce biofilms. A positive control (*L. monocytogenes* ATCC 7644) was also used. Based on the OD values obtained for NC and bacterial samples, the isolates were classified as weak ($OD_{NC} < OD \leq 2xOD_{NC}$), moderate ($2xOD_{NC} < OD \leq 4xOD_{NC}$) or strong ($4xOD_{NC} < OD$) biofilm-producers, according to Stepanović et al. (2000).

2.3. Biofilm formation assays on stainless steel

The ability to produce biofilms on stainless steel was evaluated by epifluorescence microscopy using calcofluor white dye (Sigma-Aldrich, Saint Louis, MO), as recommended by Shanks et al. (2005). Stainless steel coupons (1.0 × 1.0 cm) were placed in the bottom of wells of a 24-well flat-bottomed plastic microplate (TPP, Trasadingen, Switzerland), and 2.0 mL of each TSB bacterial suspension (nearly 10^8 cells mL^{-1}) were pipetted in each series of three wells. After incubation at 35 ± 0.5 °C for 48 h without stirring, stainless steel coupons were removed from the wells with sterile forceps, rinsed 5 times with 3 mL of sterile water, placed on a glass slide and allowed to dry at room temperature. One drop of calcofluor solution (0.05%, m/v) was added in each of the surfaces. The visualization of biofilms on stainless steel coupons was performed by using an optical epifluorescence microscope (Olympus, Tokyo, Japan), with the wavelength set at 430 nm. *L. monocytogenes* (ATCC 7644) strain was used as positive control, and served as the basis for image analysis using computer software (DP2-BSW, version 2.1; Olympus, Tokyo, Japan).

3. Results and discussion

Table 2 shows the ability of the 85 *L. monocytogenes* isolates to produce biofilm in the polystyrene microplate assays. All isolates produced biofilms on microplates, which were classified as weak ($n = 40$), moderate ($n = 41$) or strong ($n = 4$) biofilm-producers. These results are a cause for concern because *L. monocytogenes* biofilms in production lines pose serious public health risks, especially in ready-to-eat products (Jackson et al., 2011), and because contamination during processing is the primary source of this pathogen in several products such as cheese (Melo et al., 2015). The ability of *L. monocytogenes* strains to produce biofilms has been demonstrated at temperatures from 12 °C, 20 °C, 30 °C to 37 °C, with increasing biofilm formation with increasing temperatures (Kadam et al., 2013). Oliveira, Brugnera, Alves, and Piccoli (2010) found that the number of surface-adhered cells of *L. monocytogenes* on stainless steel remained constant after 48 h up to 192 h incubation at 37 °C. However, the incubation temperature should be in accordance with the natural environment in which the strains were isolated. For this reason, in the present work, the biofilm-formation assays were conducted at 35 °C, which is close to typical average temperatures (>30 °C)