

Different methods to quantify *Listeria monocytogenes* biofilms cells showed different profile in their viability

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Abstract

Listeria monocytogenes is a foodborne pathogen able to adhere and to form biofilms in several materials commonly present in food processing plants. The aim of this study was to evaluate the resistance of *Listeria monocytogenes* attached to abiotic surface, after treatment with sanitizers, by culture method, microscopy and Quantitative Real Time Polymerase Chain Reaction (qPCR). Biofilms of *L. monocytogenes* were obtained in stainless steel coupons immersed in Brain Heart Infusion Broth, under agitation at 37 °C for 24 h. The methods selected for this study were based on plate count, microscopic count with the aid of viability dyes (CTC-DAPI), and qPCR. Results of culture method showed that peroxyacetic acid was efficient to kill sessile *L. monocytogenes* populations, while sodium hypochlorite was only partially effective to kill attached *L. monocytogenes* ($p < 0.05$). When, viability dyes (CTC/DAPI) combined with fluorescence microscopy and qPCR were used and lower counts were found after treatments ($p < 0.05$). Selective quantification of viable cells of *L. monocytogenes* by qPCR using EMA revealed that the pre-treatment with EMA was not appropriate since it also inhibited amplification of DNA from live cells by ca. 2 log. Thus, the use of CTC counts was the best method to count viable cells in biofilms.

Key words: biofilms, *L. monocytogenes*, qPCR, peroxyacetic acid, sodium hypochlorite.

Introduction

Listeria monocytogenes is a Gram-positive foodborne pathogen that causes listeriosis in humans. Food contamination by *L. monocytogenes* may occur due to their ability to attach and form biofilm on stainless steel and other surfaces (Farber and Peterkin 1991; Moltz and Martin 2005; Harvey *et al.*, 2007; Shi and Zhu 2009; Winkelstroter *et al.*, 2014).

The attachment of bacteria with subsequent development of biofilms on food industry surfaces has important consequences. The occurrence of such structured microbial communities in food processing plants represents a reservoir of microorganisms and serves as a potential source of contamination of raw materials and processed products (Winkelstroter *et al.*, 2014). Biofilms are difficult to remove and may require additional physical and chemical mechanisms to reduce their presence and occurrence

(Manios and Skandamis, 2014). It is known that *L. monocytogenes* can survive sanitization procedures. Among the chemical sanitizers used in food industry, chlorine and peroxyacetic acid, which were used in this study, are considered the most popular and traditional sanitizer (Manios, Skandamis, 2014; Neo *et al.*, 2013).

Bacterial populations in biofilms are normally enumerated by detachment of cells followed by agar plating count. However, accurate quantification of bacteria in biofilms may be difficult due to the possible presence of cells in the viable but non-cultivable (VBNC) state (Besnard *et al.*, 2000; Maukonen *et al.*, 2000; Chae and Scraft 2001). VBNC cells can be detected by double staining with chemicals such as 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and 4, 6-diamidino-2-phenylindole (DAPI) because these chemicals differentiate live and dead cells and can be used combined to cells counting under fluorescence microscopy (Besnard *et al.*, 2000; Maukonem *et al.*, 2000).

Real Time Polymerase Chain Reaction (qPCR) is other method for biofilm quantification, but presents the drawback of not discriminating DNA from live and dead bacterial cells (Besnard *et al.*, 2000; Guilbald *et al.*, 2005; Nocker *et al.*, 2006). To avoid false positive results in qPCR analysis, some authors recommended the pre-treatment of samples with ethidium bromide monoazide (EMA) to inhibit selectively the amplification of DNA from dead cells (EMA qPCR) (Rudi *et al.*, 2005; Nocker *et al.*, 2006; Minami *et al.*, 2010).

The aim of this study was to evaluate the viability of *L. monocytogenes* biofilms after treatment with sanitizers using plate count, staining with viability dyes combined with fluorescence microscopy and qPCR.

Material and Methods

Bacterial strain

L. monocytogenes ATCC 19115 was used in this study and stock cultures were kept in Brain Heart Infusion broth (BHI, Oxoid, Basingstoke, UK) with 20% (v/v) glycerol (Synth, Brazil) at -80 °C. Working cultures were prepared by inoculation of 1% (v/v) of stock cultures of *L. monocytogenes* in BHI and incubation at 37 °C/24 h.

Biofilm formation

Stainless steel coupons (7.5 x 2.0 x 0.2 cm, AISI 340) were pre-treated as described by Minei *et al.* (2008), set vertically in a round support, placed in a beaker, covered with aluminum foil, and sterilized in autoclave at 121 °C for 15 min.

Approximately 800 mL of BHI broth were added to a beaker containing the clamped stainless steel coupons, *L. monocytogenes* was inoculated at ca. 10^8 UFC/mL, and this system was incubated at 37 °C for 24 h, under constant agitation at 15 rpm (Fisatom, São Paulo, Brazil). At the end of incubation, stainless steel coupons were aseptically removed from the support, followed by flooding the biofilm slide in peroxyacetic acid (1:40 v/v) solution for 3 min. or in sodium hypochlorite solution (140 ppm) for 3 min (Ratti *et al.*, 2010). After treatment with peroxyacetic acid and sodium hypochlorite, sanitizers were neutralized by covering the slides with sodium thiosulfate solution (1% w/v, Merck, Germany) for 3 min.

In this study, to consider that a biofilm was formed, at least 10^3 adhered cells per cm^2 should be quantified (Minei *et al.*, 2008).

Enumeration on agar plates

After sanitizer treatment, the coupon was rinsed with 20 mL of Phosphate Buffered Saline – PBS (pH 7.4) to remove non-adherent *L. monocytogenes* cells. Next, adhered cells were removed by manually rubbing the upper surface (15 cm^2) of the coupon with a sterile cotton swab ca. 100 times and it was transferred to a test tube containing 10 mL

of PBS. Serial dilutions were prepared and 100 μL of each dilution were surface plated on Tryptic Soy agar supplemented with 6 $\text{g}\cdot\text{L}^{-1}$ of yeast extract (TSAYE, Oxoid, UK) and incubated for 37 °C/24 h. Results were expressed as CFU per cm^2 (Minei *et al.*, 2008).

Double staining with CTC-DAPI combined with observation by fluorescence microscopy. These experiments were done based on Bredholt *et al.* (1999), with modifications. For that, the surface of the coupon was flooded with 2 mL of 5 mM CTC (Polysciences, Warrington, PA, USA) and it was kept in the dark for 2 h at 30 °C. The staining solution was drained and the biofilm was fixed for 30 min with 2 mL of formalin solution - 4% (v/v) formaldehyde (Synth, São Paulo, Brazil). DAPI 1 $\mu\text{g}/\text{mL}$ (Sigma-Aldrich, St. Louis, USA) was added to the surface of the slides and left for 20 min at room temperature. The coupon was rinsed with PBS, a cover slip was placed on the top of the slide and it was observed using an Axioskop fluorescence microscope (Zeiss, Oberkochen, Germany) under 1000 magnification with immersion oil. The total number of cells (red plus green) and the number of living cells (red) were counted from 10 random fields of $1.9 \times 10^{-3} \text{ cm}^2$ each. The results were expressed as the number of bacteria per cm^2 .

Real Time PCR quantification

For studying *L. monocytogenes* in biofilms by qPCR, coupons were removed from biofilm system, rinsed with 20 mL of PBS and adherent cells were removed from both sides of the coupons (30 cm^2) with the aid of a sterilized swab. The swab was immersed in 3 mL of PBS and the DNA extraction was performed as described by Jothikumar *et al.* (2003). The amount of CFU equivalents of DNA present in the PCR reaction tube was calculated according to De Martinis *et al.* (2007), assuming a genome content of 1 molecule per CFU.

The primers used in this study were obtained from Invitrogen (Carlsbad, USA) and they specifically detected *L. monocytogenes* (L-1:5'-CACGTGCTACAATGGATAG-3' and L-2:3'-GATTAGGGTATTTTGATAAGA-5'). They were previously designed to amplify a 70-bp sequence from the gene encoding 16S rRNA of *L. monocytogenes* (Wang *et al.*, 1992). The amplification was performed using a real-time PCR system MiniOpticon (Bio-Rad Laboratories, Hercules, CA, USA) equipped with the Opticon Monitor Analysis Software - version 3.1 (Bio-Rad Laboratories) for data acquisition and analysis of results. All PCR amplifications were performed in 25- μL reaction volume with 4 μL of culture lysate (target DNA), 12.5 μL AbsoluteM SYBR Green QPCR Master Mix (ABgene, Surrey, UK), 2.5 μL of each primer solution (0.25 mM of each primer) and 6 μL of purified water. The conditions for PCR reaction comprised an initial denaturation step at 95 °C for 15 min and 45 cycles of 95 °C for 2 s, 55 °C for 10 s, and 72 °C for 10 s. The thermocycling pro-

gram was followed by an additional heating step done at 0.3 °C/s, from 65 °C to 90 °C, to obtain the melting curve. Specificity of amplification was confirmed by agarose gel electrophoresis and the results were evaluated according to Wang *et al.* (1992) and De Martinis *et al.* (2007).

The threshold cycle (Ct) values obtained were plotted against the calculated number of DNA copies to obtain an analytical curve for quantification of DNA in samples from *L. monocytogenes* biofilms (Guilbald *et al.*, 2005).

For preventing amplification of DNA from dead cells, the use of EMA prior to qPCR was also evaluated. Biofilm or planktonic cells were obtained as previously described, the tubes were added of EMA (Sigma-Aldrich, St. Louis, USA) for a final concentration of 25 µg/mL (Rudi *et al.*, 2005; Lee and Levin 2006) and kept in the dark for 5 min. Uncapped tubes were cooled on ice and exposed to halogen lamp (500 W) (FLC, São Paulo, Brazil) for 5 min at a 20 cm distance (Rudi *et al.*, 2005; Lee and Levin 2006). Bacterial cells were centrifuged at 12,000 g for 1 min, the pellet was resuspended in 100 µL of sterilized water (Milli-Q, Millipore, USA), and lysed as described by Jothikumar *et al.* (2003). Controls were run with planktonic cells of *L. monocytogenes* non-heat treated or heat treated for 10 min prior to qPCR amplification.

Statistical analysis

Results were expressed as means ± standard deviations of at least three independent experiments and biofilm formation in BHI broth was used as control. One-way ANOVA followed by Bonferroni test was applied to verify

significant differences ($p < 0.05$) among methods and conditions (Sigma Stat 3.1-Systat Software, Richmond, CA, USA).

Results and Discussion

Sanitizers and cleaners such as peroxyacetic acid and sodium hypochlorite have been used to obtain good manufacturing practices regimes to prevent the accumulation of microbial cells and consequent biofilm formation in the food industry (Dunne 2002; Belessi *et al.*, 2011; Cruz and Fletcher, 2012; Manios, Skandamis, 2014; Winkelstroter *et al.*, 2014).

In the present study, plate count method revealed that treatments with sodium hypochlorite and peroxyacetic acid (respectively, 2.83 log cfu/cm² and counts below the detection limit of the method 1.2 log cfu/cm²) were efficient to reduce *L. monocytogenes* population in biofilms formed when compared to results for biofilm grown in BHI broth without treatment (control) ($p < 0.05$) (Figure 1). A decrease was also observed in the *L. monocytogenes* biofilm cells count by CTC staining after exposure to sodium hypochlorite and peroxyacetic acid (respectively, 1.6 and 2.1 log cfu/cm² counts) ($p < 0.05$). However, qPCR results indicated that only sodium hypochlorite treatment was efficient to reduce the biofilm cell counts in comparison with the cells obtained in BHI broth (control) ($p < 0.05$).

Results on the efficacy of disinfectants are in agreement with results found using planktonic cells instead of biofilm cells (Park *et al.*, 2012). Also, Belessi *et al.* (2011)

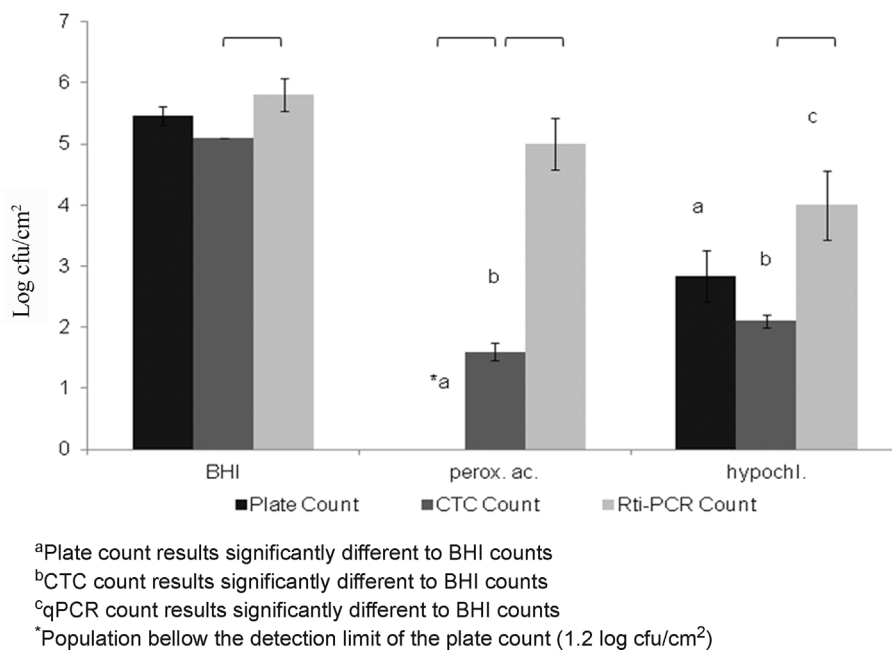


Figure 1 - Population of *L. monocytogenes* adhered on stainless steel coupons enumerated by plate, direct fluorescent microscopy with CTC-DAPI staining and qPCR: BHI) Brain Heart Infusion (BHI) broth; perox. ac.) BHI broth + treatment with peroxyacetic acid (1:40 v/v); hypochl.) BHI broth + treatment with sodium hypochlorite solution 140 ppm. Results are means of triplicates and vertical bars show standard deviations. Brackets indicate statistic difference among methods used.

and Mittelman (1997) found that the number of cells forming the biofilm of *L. monocytogenes* in stainless steel coupons decreased as the exposure time to 2% peroxyacetic acid increased leading to no detection of biofilm cells after 6 min of exposure. The authors also reported that low concentrations of sodium hypochlorite, in the range of 0.5 to 5 ppm, were only inhibitory to biofilms adhered to stainless steel surfaces. However, despite the good effectiveness of peroxyacetic acid, the length of treatment and sanitizer concentration applied must be adequate for inactivating *L. monocytogenes* and it is also important to note that food matrix components can change the activity of the sanitizer (Aarnisalo *et al.*, 2007; Gram *et al.*, 2007; Manios and Skandamis, 2014).

The comparison between methods showed that counts obtained with CTC staining were significant higher if compared to plate agar count, after treatment with peroxyacetic acid, probable due to the presence of VBNC cells that were not detected by culture method. On the other hand, qPCR presented significantly higher counts in comparison to CTC counts (both control and treated samples). Therefore, these results confirm that the qPCR method only is not able to discriminate DNA from live and from dead bacterial cells and could lead to false positive results (Besnard *et al.*, 2000; Guilbald *et al.*, 2005; Nocker *et al.*, 2006).

Combination of EMA with qPCR was not an adequate tool for quantifying viable cells of *L. monocytogenes*, since EMA inhibited DNA amplification of planktonic *L. monocytogenes* cells by ca. 2 log cfu/mL (data not shown) regardless if cells had been submitted or not to heat treatment (10^7 vs. 10^9). Similarly, Flekna *et al.* (2007) showed that after EMA treatment, populations of *L. monocytogenes* by qPCR were also underestimated. Improvement and further standardization of these methods are necessary to avoid loss of DNA from viable cells and to permit the selective quantification of viable organisms.

Conclusion

Overall, our findings demonstrated that some sanitizers can be used to reduce biofilms. Also, this study highlighted the importance of the method used to quantify the cells since by plate count the viable *L. monocytogenes* population in biofilms can be underestimated and by qPCR could generate false-positive results. The attempt of using EMA pre-treatment in PCR reaction was unsuccessful because it also inhibited the amplification of viable cells. Thus, we concluded that the use of CTC counts was the best choice method to count viable cells in biofilms.

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References

- Aarnisalo K, Lunden J, Korkeala H *et al.* (2007) Susceptibility of *Listeria monocytogenes* strains to disinfectants and chlorinated alkaline cleaners at cold temperatures. *LWT-Food Sci Technol* 40:1041-1048.
- Belessi CEA, Gounadaki AS, Psomas AN *et al.* (2011) Efficiency of different sanitation methods on *Listeria monocytogenes* biofilms formed under various environmental conditions. *Int J Food Microbiol* 145:S46-S52.
- Besnard V, Federighi M, Cappelier JM (2000) Evidence of viable but non-culturable state in *Listeria monocytogenes* by direct viable count and CTC-DAPI double staining. *Food Microbiol* 17:697-704.
- Bredholt S, Maukonen J, Kujanpaa K *et al.* (1999) Microbial methods for assessment of cleaning and disinfection of food-processing surfaces cleaned in a low-pressure system. *Eur Food Res Technol* 209:145-152.
- Chae MS, Schraft H (2001) Cell viability of *Listeria monocytogenes* biofilms. *Food Microbiol* 18:103-112.
- Cruz CD, Fletcher GC (2012) Assessing manufacturers' recommended concentrations of commercial sanitizers on inactivation of *Listeria monocytogenes*. *Food Control* 26:194-200.
- De Martinis ECP, Duvall RE, Hitchins AD (2007) Real-time PCR detection of 16S rRNA genes speeds most-probable-number enumeration of foodborne *Listeria monocytogenes*. *J. Food Prot.* 70:1650-1655.
- Dunne WM (2002) Bacterial adhesion: Seen any good biofilms lately? *Clin Microbiol Rev* 15:155-166.
- Farber JM, Peterkin PI (1991) *Listeria monocytogenes*, a food-borne pathogen. *Microbiol Rev* 55:476-511.
- Flekna G, Stefanic P, Wagner M *et al.* (2007) Insufficient differentiation of live and dead *Campylobacter jejuni* and *Listeria monocytogenes* cells by ethidium monoazide (EMA) compromises EMA/real-time PCR. *Res Microbiol* 158:405-412.
- Gram L, Bagge-Ravn D, Ng YY *et al.* (2007) Influence of food soiling matrix on cleaning and disinfection efficiency on surface attached *Listeria monocytogenes*. *Food Control* 18:1165-1171.
- Guilbald M, Coppet P, Bourion F *et al.* (2005) Quantitative detection of *Listeria monocytogenes* in biofilms by real-time PCR. *Appl Environ Microbiol* 71:2190-2194.
- Harvey J, Keenan KP, Gilmour A (2007) Assessing biofilm formation by *Listeria monocytogenes* strains. *Food Microbiol* 24:380-392.
- Jothikumar N, Wang XW, Griffiths MW (2003) Real-time multiplex SYBR green I-based PCR assay for simultaneous detection of *Salmonella* serovars and *Listeria monocytogenes*. *J Food Prot* 66:2141-2145.
- Lee JL, Levin RE (2006) Use of ethidium bromide monoazide for quantification of viable and dead mixed bacterial flora from fish fillets by polymerase chain reaction. *J Microbiol Methods* 67:456-462.
- Manios SG, Skandamis PN (2014) Control of *Listeria monocytogenes* in the Processing Environment by Under-

- standing Biofilm Formation and Resistance to Sanitizers. *Methods Mol Biol* 1157:251-261.
- Maukonen J, Mattila-Sandholm T, Wirtanen G (2000) Metabolic indicators for assessing bacterial viability in hygiene sampling using cells in suspension and swabbed biofilm. *LWT-Food Sci Technol* 33:225-233.
- Minami J, Yoshida K, Soejima T *et al.* (2010) New approach to use ethidium bromide monoazide as an analytical tool. *J Appl Microbiol* 109:900-909.
- Minei CC, Gomes BC, Ratti RP *et al.* (2008) Influence of peroxyacetic acid and nisin and coculture with *Enterococcus faecium* on *Listeria monocytogenes* biofilm formation. *J Food Prot* 71:634-638.
- Mittelman MW (1998) Structure and functional characteristics of bacterial biofilms in fluid processing operations. *J Dairy Sci* 81:2760-2764.
- Moltz AG, Martin SE (2005) Formation of biofilms by *Listeria monocytogenes* under various growth conditions. *J Food Prot* 68:92-97.
- Neo SY, Lim PY, Phua LK *et al.* (2013) Efficacy of chlorine and peroxyacetic acid on reduction of natural microflora, *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella* spp. on mung bean sprouts. *Food Microbiol* 36:475-80.
- Nocker A, Cheung CY, Camper AK (2006) Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. *J Microbiol Methods* 67:310-320.
- Park S-H, Cheon H-L, Park K-H *et al.* (2012) Inactivation of biofilm cells of foodborne pathogen by aerosolized sanitizers. *Int J Food Microbiol* 154:130-134.
- Ratti RP, Gomes BC, Martinez RCR *et al.* (2010) Elongated cells of *Listeria monocytogenes* in biofilms in the presence of sucrose and bacteriocin-producing *Leuconostoc mesenteroides*. *A11 Ciênc Tecnol Aliment* 30:1011-1016.
- Rudi K, Naterstad K, Dromtorp SM *et al.* (2005) Detection of viable and dead *Listeria monocytogenes* on gouda-like cheeses by real-time PCR. *Lett Appl Microbiol* 40:301-306.
- Shi XM, Zhu XN (2009). Biofilm formation and food safety in food industries. *Trends Food Sci Technol* 20:407-413.
- Wang RF, Cao WW, Johnson MG (1992) 16S ribosomal-RNA-based probes and polymerase chain-reaction method to detect *Listeria monocytogenes* cells added to foods. *Appl Environ Microbiol* 58:2827-2831.
- Winkelstroter LK, Reis FB, Silva EP *et al.* (2014) Unraveling Microbial Biofilms of Importance for Food Microbiology. *Microb Ecol* 68:35-46.

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