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Impact of environmental factors on the culturability and viability of *Listeria monocytogenes* under conditions encountered in food processing plants

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ABSTRACT

The ability of *Listeria monocytogenes* to adhere to and persist on surfaces for months or even years may be responsible for its transmission from contaminated surfaces to food products. Hence the necessity to find effective means to prevent the establishment of *L. monocytogenes* in food processing environments. The aim of this study was to assess, through a fractional experimental design, the environmental factors that could affect the survival of *L. monocytogenes* cells on surfaces to thereby prevent the persistence of this pathogen in conditions mimicking those encountered in food processing plants: culture with smoked salmon juice or meat exudate, use of two materials with different hygiene status, biofilm of *L. monocytogenes* in pure-culture or dual-culture with a *Pseudomonas fluorescens* strain, application of a drying step after cleaning and disinfection (C&D) and comparison of two strains of *L. monocytogenes*. Bacterial survival was assessed by culture, qPCR to quantify total cells, and propidium monoazide coupled with qPCR to quantify viable cells and highlight viable but non-culturable (VBNC) cells. Our results showed that failure to apply C&D causes cell persistence on surfaces. Moreover, the sanitation procedure leads only to a loss of culturability and appearance of VBNC populations. However, an additional daily drying step after C&D optimises the effectiveness of these procedures to reduce culturable populations. Our results reinforce the importance to use molecular tools to monitor viable pathogens in food processing plants to avoid underestimating the amounts of cells using only methods based on cell culture.

1. Introduction

Listeria monocytogenes is a zoonotic pathogen causing the rare but serious listeriosis (Donovan, 2015). This foodborne disease primarily affects the developing fetus during pregnancy and immunosuppressed individuals, leading to death in 15–30% of cases (Goulet et al., 2012; Rocourt, 1996). Outbreaks are generally due to consumption of ready-to-eat products (Rocourt et al., 2003; Tompkin, 2002), with contamination occurring throughout the food chain. *L. monocytogenes* is in fact distributed in many environments, including food processing plants.

To eliminate this pathogen in factories, cleaning and disinfection (C&D) procedures are performed daily and are some of the most important measures for *L. monocytogenes* risk control (Jessen and Lammert, 2003; Rotariou et al., 2014). However, failure of C&D procedures have been reported to result in cases of survival of *L. monocytogenes* in several food processing environments causing the apparition of persistent strains (Autio et al., 2003; Ferreira et al., 2014; Lundén et al., 2003b; Møretrø and Langsrud, 2004; Tompkin, 2002; Wulff et al., 2006). Persistence can be defined as the repeated isolation of organisms of a specific molecular subtype from the same processing environment despite the frequent and correct application of C&D procedures (Carpentier and Cerf, 2011; Keto-Timonen et al., 2007).

Thus, strains of *L. monocytogenes* can remain on equipment or surfaces in niches (Mettler and Carpentier, 1999) for several months or years (Carpentier and Cerf, 2011; Jessen and Lammert, 2003). These strains may be protected by retention areas and thus exposed to sub-lethal concentrations of sanitisers. What is of concern is that the presence of persistent cells on food-contact surfaces can be a source of recontamination (Lundén et al., 2002; Reij and Den Aantrekker, 2004). The risk of cross-contamination by transfer of cells from surfaces and equipment to food products is a major threat to the food industry and consumers.

One hypothesis to explain persistence is the ability of bacteria to adapt to and survive environmental stresses such as nutrient deprivation, heat or cold temperatures, sanitisers and preservatives, desiccation, low pH, and high salt concentrations (Carpentier and Cerf, 2011; Melo et al., 2015; Thévenot et al., 2006). This ability to persist makes contamination difficult to control and eradicate. Elimination is even more difficult as cells adhere to several materials found in food processing environments, where they grow and form biofilms (Donlan, 2002; Møretrø and Langsrud, 2004; Pereira da Silva and Pereira De Martinis, 2013; Ronner and Wong, 1993). Microbial populations growing in biofilms can be difficult to destroy because they often have greater

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disinfectant resistance than planktonic organisms (Nakamura et al., 2013; Pan et al., 2006). Consequently, it is important to find effective means to prevent the establishment of *L. monocytogenes* in food processing environments to avoid biofilm formation and bacterial persistence.

Moreover, the multitude of adverse environmental conditions in food processing environments can lead to the appearance of viable but non-culturable (VBNC) cells that have lost their ability to form colonies on conventional culture media but remain alive (Li et al., 2014a; Oliver, 2005). Given that detection of *L. monocytogenes* in surface samples from food processing environments is based only on conventional microbiological methods, VBNC cells represent a real risk because they are not detected but are potentially a source of contamination of food products.

The aim of this study was to test environmental factors commonly found in food processing plants to determine their impact on the survival and persistence of *L. monocytogenes* cells. Environmental factors included (i) comparison of two different strains of *L. monocytogenes* strongly and weakly resistant to desiccation, (ii) use of two surface materials with a different hygiene status for biofilm formation, (iii) biofilm of *L. monocytogenes* in pure-culture biofilm or in dual-culture biofilm with a *Pseudomonas fluorescens* strain that promotes its adhesion, (iv) use of smoked salmon juice or meat exudate as culture medium, and (v) application of a drying step daily for a week or only once a week after C&D. For this purpose, sessile cells were subjected daily for a week to sanitation procedure and cultured with a food soil simulating in the laboratory the alternation of C&D and production steps. To study the impact of these environmental factors on the survival of *L. monocytogenes*, bacterial concentration evolution (the initial population compared to the remaining population after sanitation procedures applied daily for a week) was assessed by cell culture, quantitative PCR (qPCR) to quantify total cells, and propidium monoazide (PMA) coupled with qPCR (PMA-qPCR) to quantify viable cells and highlight VBNC cells.

2. Experimental procedures

2.1. Bacterial strains

Two strains of *L. monocytogenes* were used: LO28 (human isolate, 1/2c serotype) and EGD-e (animal isolate, 1/2a serotype). For dual-culture biofilms, *L. monocytogenes* was cultured in presence of the *Pseudomonas fluorescens* 09empf87 strain, which enhances the adhesion of *L. monocytogenes* on the surface (unpublished results).

2.2. Preparation of food soils

Smoked salmon juice and meat exudate were prepared as previously described (Midelet and Carpentier, 2004; Overney et al., 2016). Prior to use, the required volume of food soil was thawed, centrifuged (2100*g*, 10 min, 20 °C) and the supernatant was then filter-sterilised through a 0.22-μm-pore-size Stericup filter with a 2-μm-pore-size prefilter (Millipore, Saint-Quentin-en-Yvelines, France).

The meat exudate contained 9.93 g/100 g protein and less than 0.1 g/100 g lipid. The smoked salmon juice contained 1.93 g/100 g protein and 0.14 g/100 g lipid (ISHA, Institut Scientifique d'Hygiène et d'Analyse, Longjumeau, France).

2.3. Culture conditions

Long-shelf-life stock cultures of the strains used in this study were stored at −80 °C. They were used to inoculate tryptone soya agar (TSA, Biomérieux, Bruz, France) slopes incubated for 24 h at 25 °C. These were then maintained at 4 °C for no more than one month until use. The day before each experiment, the refrigerated culture was transferred to another TSA slope and incubated for 24 h at 25 °C.

Two protocols were followed according to the food soil used. For pure-culture biofilm experiments with smoked salmon juice, *L. monocytogenes* cells were suspended in TSB/5_m, which is the culture medium developed to mimic this food soil (Overney et al., 2016). The optical density (OD) at 600 nm of the suspension was adjusted to 1.5 (corresponding to a concentration of about 10^9 CFU/ml) in a 1.5-cm-diameter tube. This was the bacterial suspension used for biofilm formation. For pure-culture biofilm experiments with meat exudate, bacteria were suspended in 1 g/l peptone water (AES Chemunex, Bruz, France) and the OD adjusted as described above. The volume of the suspension necessary for the experiment was then centrifuged at 2100*g* for 10 min at room temperature, the supernatant removed, and the pellet suspended in the same volume of filter-sterilised meat exudate.

For dual-culture biofilm experiments, bacterial suspensions were prepared differently. *P. fluorescens* cells were suspended in 1 g/l peptone water, the OD adjusted to 1.5, and then diluted to 1:100 (corresponding to a concentration of about 10^7 CFU/ml) in smoked salmon juice or in meat exudate. *L. monocytogenes* cells were suspended in 1 g/l peptone water, the OD adjusted to 0.15, and then diluted to 1:1000 (corresponding to a concentration of about 10^5 CFU/ml) in smoked salmon juice or in meat exudate.

2.4. Biofilm development

Biofilms were prepared on coupons (4 cm by 2.5 cm) in stainless steel (SS, 2 RB finish, AISI 304, Laser 53, Bazougers, France) or in ceramic clinker tiles dry-pressed unglazed (Argelith Bodenkeramik, Bad Essen, Germany). SS coupons were rubbed with a mixture of ethanol at 95% and acetone (3 V/1 V) to remove any manufacturing process debris and grease. As previously described by Peneau et al. (2007), a silicon joint sealant (Fischer, France) was applied along the edges of each coupon to obtain an area than could be kept flooded for experiments. Each SS and ceramic coupon was then washed and autoclaved (Aase et al., 2000; Leriche and Carpentier, 1995). Lastly, SS surfaces were placed in 90-mm-diameter Petri dishes. To prevent dehydration of biofilms during incubation, each 90-mm-diameter Petri dish was then placed in a 120-mm-diameter Petri dish containing 25 ml of sterile water. Ceramic coupons were placed into sterilised boxes measuring 26 cm by 15 cm containing 50 ml of sterile water.

For pure-culture biofilms on ceramic and SS surfaces, 1 ml of the *L. monocytogenes* suspension prepared as described above was left on clean coupons. These were incubated at 25 °C for 3 h to allow adhesion of bacteria. Then, the non-adherent bacteria were removed by pouring 25 ml of 1 g/l peptone water over the bacterial deposit. A volume of 1 ml of filter-sterilised food soil was deposited so as to cover sessile cells. Lastly, coupons were incubated for 21 h at 25 °C.

For dual-culture biofilms on ceramic and SS surfaces, 1 ml of the *P. fluorescens* suspension prepared as described above was deposited on coupons. After incubation at 25 °C for 3 h, the non-adherent cells were removed as described above. Then, 1 ml of the *L. monocytogenes* suspension was left. The coupons were then incubated for 21 h at 25 °C.

2.5. Repeated soiling and chemical treatments of the coupons

Repeating soiling and C&D procedures were applied without mechanical action (Marouani-Gadri et al., 2010; Peneau et al., 2007). The total duration of the experiments was 4 days taking as the starting point the day on which the first C&D was performed. Topax 66 (Ecolab, Arcueil, France) is a chlorinated alkaline cleaner used at a concentration of 3% (vol/vol). Triquart MS (Ecolab) is a quaternary ammonium-based disinfectant with a recommended concentration of 2% (vol/vol). Both chemical products are frequently used in the meat and fish industries. To obtain surviving populations on SS surfaces and then to study the impact of parameters on the survival of *L. monocytogenes*, Topax 66 and Triquart MS were diluted to model what occurs in retention zones that cannot be reached by mechanical action of cleaning and where some water remains before product application (Marouani-Gadri et al., 2010; Peneau et al., 2007).