International Journal of Food Microbiology 267 (2018) 9–19

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

Dynamics of biofilm formation by *Listeria monocytogenes* on stainless steel under mono-species and mixed-culture simulated fish processing conditions and chemical disinfection challenges

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ARTICLE INFO

Keywords:
Listeria monocytogenes
Biofilm
Stainless steel
Seafood
Pseudomonas spp.
Disinfection

ABSTRACT

The progressive ability of a six-strains *L. monocytogenes* cocktail to form biofilm on stainless steel (SS), under fish-processing simulated conditions, was investigated, together with the biocide tolerance of the developed sessile communities. To do this, the pathogenic bacteria were left to form biofilms on SS coupons incubated at 15 °C, for up to 240 h, in periodically renewable model fish juice substrate, prepared by aquatic extraction of sea bream flesh, under both mono-species and mixed-culture conditions. In the latter case, *L. monocytogenes* cells were left to produce biofilms together with either a five-strains cocktail of four *Pseudomonas* species (*fragi*, *savastanoi*, *putida* and *fluorescens*), or whole fish indigenous microflora. The biofilm populations of *L. monocytogenes*, *Pseudomonas* spp., Enterobacteriaceae, H_2S producing and aerobic plate count (APC) bacteria, both before and after disinfection, were enumerated by selective agar plating, following their removal from surfaces through bead vortexing. Scanning electron microscopy was also applied to monitor biofilm formation dynamics and anti-biofilm biocidal actions. Results revealed the clear dominance of *Pseudomonas* spp. bacteria in all the mixed-culture sessile communities throughout the whole incubation period, with the in parallel sole presence of *L. monocytogenes* cells to further increase (ca. 10-fold) their sessile growth. With respect to *L. monocytogenes* and under mono-species conditions, its maximum biofilm population (ca. 6 log CFU/cm^2) was reached at 192 h of incubation, whereas when solely *Pseudomonas* spp. cells were also present, its biofilm formation was either slightly hindered or favored, depending on the incubation day. However, when all the fish indigenous microflora was present, biofilm formation by the pathogen was greatly hampered and never exceeded 3 log CFU/cm^2, while under the same conditions, APC biofilm counts had already surpassed 7 log CFU/cm^2 by the end of the first 96 h of incubation. All here tested disinfection treatments, composed of two common food industry biocides gradually applied for 15 to 30 min, were insufficient against *L. monocytogenes* mono-species biofilm communities, with the resistance of the latter to significantly increase from the 3rd to 7th day of incubation. However, all these treatments resulted in no detectable *L. monocytogenes* cells upon their application against the mixed-culture sessile communities also containing the fish indigenous microflora, something probably associated with the low attached population level of these pathogenic cells before disinfection ($< 10^2$ CFU/cm^2) under such mixed-culture conditions. Taken together, all these results expand our knowledge on both the population dynamics and resistance of *L. monocytogenes* biofilm cells under conditions resembling those encountered within the seafood industry and should be considered upon designing and applying effective anti-biofilm strategies.

1. Introduction

L. monocytogenes is a significant foodborne bacterial pathogen provoking listeriosis, a relatively rare but still life-threatening disease in immunocompromised individuals (Gandhi and Chikindas, 2007). In Europe, 2161 confirmed human listeriosis cases and 210 deaths were recorded in 2014 (EFSA and ECDC, 2015). Alarmingly, this year, the hospitalization and case-fatality ratios were 98.9% and 15%, respectively. *L. monocytogenes* can adhere to and produce biofilms on many surfaces encountered within the food industry (Valderrama and Cutter, 2013), resulting in potential continuous contamination of the products (Hansen and Vogel, 2011; Tresse et al., 2007). These multicellular sessile microbial communities are embedded in hydrated extracellular polymeric matrixes and their formation is more and more considered as

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https://doi.org/10.1016/j.ijfoodmicro.2017.12.020
Received 5 September 2017; Received in revised form 4 December 2017; Accepted 17 December 2017
Available online 19 December 2017
0168-1605/

a universal microbial adaptation mechanism against various stresses and antimicrobials, like those encountered within food processing (Bridier et al., 2015). To this direction, numerous studies have shown that *L. monocytogenes* strains of different origins and serotypes, are able to produce biofilms on a variety of surfaces, both biotic and abiotic, depending on the strain, surface and culture conditions (da Silva and De Martinis, 2013; Harvey et al., 2007; Mosquera-Fernández et al., 2014; Nilsson et al., 2011; Poimenidou et al., 2016). Indeed, the great persistence this pathogen may display in food processing plants upon its enclosure in biofilm structures, even for years, is believed to seriously contribute to its transmission and be the reason behind several human listeriosis outbreaks (Carpentier and Cerf, 2011; Ferreira et al., 2014; Nowak et al., 2017).

Seafood consumption is worldwide an integral part of a healthy diet. However, this is not without risk, with seafood commodities commonly implicated in food-borne disease outbreaks, caused by a variety of bacteria, viruses and parasites (Iwamoto et al., 2010). Although *L. monocytogenes* may be present in many foods, ready-to-eat (RTE) ones, such as marinated or cold smoked fishes, require the greatest attention. This is indeed included in the common seafood bacterial pathogens, with the proportion of positive for this bacterium samples in RTE foods at European Union retail markets, in 2014, to be highest in fish products (mainly smoked fish) (EFSA and ECDC, 2015). Sea bream (*Sparus aurata*) is one of the most important cultured fish species in the Mediterranean region and especially Greece, which is the leading producer in the world with 44.3% of the total production (FAO, 2011–2017). Alarmingly, biofilm formation has been problematic in the aquaculture industry from farm (preharvest) through the processing plant (postharvest) (Mizan et al., 2015; Rajkowski, 2009). Although the prevalence of *L. monocytogenes* in European raw fish could be considered low, with an overall one of 3% to have been described (Davies et al., 2001), the fact that these bacteria are psychrotrophic and can also grow under modified atmospheres and/or high salt content is of concern (Provincial et al., 2013). The risk of infection increases considering that fish and fishery products may also sometimes be eaten undercooked.

Given this serious risk, previous research has focused on the ecology and prevalence of *L. monocytogenes* in fish processing environments (Hoffman et al., 2003; Leong et al., 2015; Nakamura et al., 2013; Thimothe et al., 2002). In such a study, all raw RTE seafood isolates of *L. monocytogenes* tested ($n = 61$) could form biofilms to various degrees (Takahashi et al., 2009). However, most of the studies on the ability of *L. monocytogenes* to colonize abiotic surfaces have been conducted under monoculture conditions, and with the use of laboratory (synthetic) growth media to support growth. Nevertheless, this does not sufficiently represent the real situation encountered in food processing environments, where *L. monocytogenes* may be present on surfaces with many other bacteria forming multi-species biofilms (Dzieciol et al., 2016; Liu et al., 2016). Under such conditions, interspecies interactions are unavoidable and may affect both the dynamics of biofilm formation by each individual species and their resistance (Giaouris et al., 2015). Thus, the potential protection of pathogenic cells by resident surface flora inside mixed-culture biofilms when subjected to disinfection is surely a matter of concern (Sanchez-Vizuete et al., 2015). An example of this is *L. monocytogenes* surviving in biofilms formed by *Pseudomonas* (Puga et al., 2016). These latter are Gram-negative aerobic bacteria which are commonly found as the main components of surface flora isolated from food processing environments following regular cleaning and disinfection (Fagerlund et al., 2017; Guobjörnsdóttir et al., 2005; Langsrud et al., 2016; Liu et al., 2016) and have been also described as good biofilm producers (Mann and Wozniak, 2012).

In food industry, conditioning films are quickly developed on various surfaces following the absorption of different types of food residues onto them (Whitehead et al., 2010). Such films can influence both microbial biofilm formation on these surfaces and in parallel the subsequent resistance of surface-attached cells to various stresses, including cleaning and disinfection, causing the failure of sanitization processes and leading to persistence. For instance, the behavior of sessile *L. monocytogenes* cells has been previously found to be affected by the type of food soil (Gram et al., 2007; Kuda et al., 2015; Overney et al., 2016; Takahashi et al., 2011). It is therefore recommended, whenever possible, to study biofilm formation and/or resistance of sessile cells by using, in the laboratory, food soils or media simulating the ones likely to be encountered by microorganisms in real food processing environments to better understand and predict their sessile behavior.

Considering all the above, the objective of the current study was to assess the progressive ability of a six-strains *L. monocytogenes* cocktail to form biofilm on stainless steel (SS), under fish-processing simulated mono-species or mixed-culture conditions, together with the biocide tolerance of the developed sessile communities against two common food industry chemical disinfectants. In the case of mixed-culture conditions, pathogenic cells were left to produce biofilms together with either a five-strains cocktail of four *Pseudomonas* species (*fragi*, *savastanoi*, *putida* and *fluorescens*), or whole fish indigenous microflora. Model fish juice substrate was always used to support sessile development.

2. Materials and methods

2.1. Bacterial strains, media and preparation of the inocula

Six *L. monocytogenes* and five *Pseudomonas* spp. strains were used in this study (Table 1). Regarding the *L. monocytogenes* strains, two were of clinical origin (FMCC_B124; FMCC_B125), two had been isolated from foods (FMCC_B127; FMCC_B128), and two from food processing environments (FMCC_B131; FMCC_B157). Regarding the *Pseudomonas* spp. strains, one belonged to *P. fragi* species (FMCC_B209), one to *P. savastanoi* species (ATCC13526), two to *P. putida* species (ATCC12633; FMCC_B74), and one to *P. fluorescens* species (FMCC_B29). All strains, apart from ATCC13526 and ATCC12633, were kindly provided by Prof. G.-J. Nychas from the Agricultural University of Athens (Athens, Greece).

Before utilization, all bacteria were stored frozen at − 80 °C in bead vials (Protect; Technical Service Consultants, Ltd., Heywood, Lancashire, United Kingdom) and were then resuscitated by adding one bead for each strain to 10 ml of Tryptone Soy Broth (TSB; Lab M; International Diagnostics Group Plc, Bury, Lancashire, UK) and incubating for 24 h at either 37 °C (*L. monocytogenes* strains) or 25 °C (*Pseudomonas* spp. strains) (precultures). These temperatures were chosen to be closely to the optimum for each genus to quickly and successfully resuscitate the bacteria following their deep-frozen storage. Working cultures were prepared by adding a 10-μl aliquot of each preculture to 10 ml of TSB and incubating for another 24 h at the appropriate temperature mentioned above. Cells from final (stationary phase) working cultures were harvested by centrifugation (2500 × *g* for 10 min at room temperature), washed twice and re-suspended in sterile saline solution (0.85% w/v NaCl). Finally, the bacterial suspensions of the six *L. monocytogenes* and/or the five *Pseudomonas* spp. strains were combined and further diluted in sterile saline solution to yield inocula of approximately 10^{7-8} CFU/ml, to be used for the subsequent biofilm formation assays. It should be noted that care was taken in order all these three inocula to contain approximately the same number of cells for each strain.

2.2. Preparation of fish juice broth (FJB) model substrate

Sterile FJB model substrate was prepared by the extraction method described by Parlapani et al. (2017). Briefly, 3 kg of fresh fish farm breams were purchased from a fish shop in Volos (Greece) and transported, in ice, to the laboratory within 1 h of purchase. There, 250 g of their flesh were aseptically cut in small pieces and homogenized with 250 ml of sterile deionized water, for 2 min, using a Stomacher (Bug