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BIOFILM FORMATION BY PERSISTENT AND NON-PERSISTENT *LISTERIA MONOCYTOGENES* STRAINS ON ABIOTIC SURFACES

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Contaminated food with *Listeria monocytogenes* is the predominant route of transmission of listeriosis to humans, a severe illness with a high mortality rate. Food processing environments can be colonized by persistent strains, repeatedly isolated for months or years. This study aimed to investigate the biofilm formation capacity of six strains of *L. monocytogenes* that persisted for long periods in two cheese processing plants in comparison to seven strains isolated sporadically from the same plants. Persistent strains produced more biofilm than non-persistent strains on stainless steel and silicon rubber surfaces; no significant differences were observed on polyvinyl chloride (PVC). In a polystyrene microtiter plate assay with crystal violet staining, no evidence was found that persistent strains have higher ability to form biofilm than non-persistent strains, and no correlation was identified between biofilm formation in the microtiter plate and on the three other surfaces tested.

Keywords: *Listeria monocytogenes*, persistence, biofilm, stainless steel, silicon, PVC

Listeria monocytogenes is a Gram-positive bacterium responsible for causing listeriosis in humans. Although it is a rare disease, with a relatively low incidence (0.52 cases of invasive listeriosis per 100 000 population in 2014), listeriosis is a severe disease in terms of hospitalization, with the highest case fatality rate (15.0%) of all the zoonotic diseases under EU surveillance (EFSA & ECDC, 2015). Contaminated food represents the major transmission route of this pathogen. Its common environmental distribution together with its unusual ability to adapt and survive under extreme conditions, as those inflicted by the environmental stresses of food processing plant, makes the development of effective strategies to control *L. monocytogenes* crucial. Cross-contamination by the equipment and general environment within the processing plant, after the foods have been processed, have been pointed out as one of the most important sources of food product contamination (Lappi et al., 2004; Almeida et al., 2013).

Several studies have demonstrated the colonization for long time periods (months or even years) of food processing plants by *L. monocytogenes* isolates presenting indistinguishable molecular subtypes (Ferreira et al., 2011; Almeida et al., 2013). These have been denominated persistent or dominant strains, while others that are recovered sporadically from the food environment are considered non-persistent or transient strains. This prolonged persistent contamination is not currently fully understood, and the hypothesis that persistent strains present distinct phenotypic traits, that allow their survival for long periods has been suggested. However, until now no strong evidence has been provided to validate this association (reviewed by Ferreira and co-workers, 2014).

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Persistence of *L. monocytogenes* in the processing environment could be possible related to a higher ability to form biofilms where cells would be protected from the environmental stresses, e.g. *L. monocytogenes* cells in biofilm present increased resistance to antimicrobial agents and disinfectants than in the planktonic forms (Norwood & Gilmour, 2000; Stopforth et al., 2002).

The importance of research directed toward the elucidation of *L. monocytogenes* persistence is a crucial step for the development of effective and practical control strategies, targeting hygienic and sanitary issues, and decrease the likelihood of cross-contamination, such as the "seek-and-destroy" approach referred by Malley and co-workers (2015).

In the present study we analysed 13 *L. monocytogenes* strains, selected from a larger set of isolates, previously characterised by different genotypic and phenotypic methods (Magalhães et al., 2016). These were randomly chosen to evaluate the ability of persistent and non-persistent *L. monocytogenes* strains to form biofilm on different abiotic surfaces, including: stainless steel, silicon rubber, and polyvinyl chloride (PVC). Biofilm formation by all strains using a microtiter plate assay was also investigated.

1. Materials and methods

1.1. Listeria monocytogenes *strains and inoculum preparation*

A set of persistent and non-persistent *L. monocytogenes* strains were selected from *Listeria* Research Centre from Escola Superior de Biotecnologia (LRCESB) culture collection based on previous study by Almeida and co-workers (2013) that evaluated during a four-year period the contamination by *L. monocytogenes* in the environment, raw material, and final-products of different cheese-processing plants. Isolates collected in that study were characterised by DNA macrorestriction analysis by pulsed field gel electrophoresis (PFGE). The recurrent isolation of *L. monocytogenes* isolates with undistinguished molecular PFGE types on different dates and origins indicated evidence of in-house persistence in an artisanal producer of raw ewe's milk cheeses (APC) and in a small-scale industrial cheese producer (SSI). For the present study, six strains were randomly selected to represent PFGE types Da, Db, and Dc (recurrently isolated during 15, 9, and 8 months, respectively) from APC producer, and PFGE types Ka, Kb, and E (recurrently isolated over a period of four, three, and four years, respectively) from SSI producer (Table 1); these were denominated persistent strains. These strains were characterised in a previous study by Magalhães and co-workers (2016), in which several isolates from each persistent PFGE type recovered from these two processing plants were evaluated concerning disinfectant resistance and kinetic behaviour at different temperatures. As no significant differences were observed among isolates belonging to the same persistent PFGE type, for this study, single strains representative of each PFGE type were selected. Additionally, seven *L. monocytogenes* strains that were isolated only once during the period of study (denominated non-persistent strains) in both processing plants were also included (Table 1).

Table 1. Characteristics of *Listeria monocytogenes* strains used in this study

Cheese producer[a]	*L. monocytogenes* strain[b]	Year	Month	Origin	Geno-serogroup[c]	PFGE Type[c]	P / NP[d]
APC	1757	2006	Feb	Cheese	IVb	Da	P
	1606	2006	Jun	Cheese	IVb	Db	P
	1728	2006	Jan	Cheese	IVb	Dc	P
	929	2004	Feb	Cheese	IIa	C	NP
	1499	2005	Feb	Cheese	IIa	B	NP
	1712	2006	Dec	CW zone, floor	IIa	A	NP
SSI	798	2003	Jun	Cheese	IIb	Ka	P
	1108	2004	May	Cheese	IIb	Kb	P
	1597	2005	May	Shipping zone, table	IIa	E	P
	930	2004	Mar	Cow's raw milk	IIa	F	NP
	994	2004	Feb	Goat's raw milk	IIa	G	NP
	747	2003	May	Cheese	IIb	Ha	NP
	1302	2004	May	Cheese washing zone, drain	IIb	M	NP

[a]: APC: artisanal producer of row ewe's milk cheeses; SSI: small-scale industrial cheese producer; [b]: *Listeria monocytogenes* strains code selected from a previous study (ALMEIDA et al., 2013); [c]: Strains subtyping by geno-serotyping and pulsed field gel electrophoresis (PFGE) was performed by ALMEIDA and co-workers (2013); geno-serogroup IVb (serotypes 4b, 4d, and 4e), geno-serogroup IIa (serotypes 1/2a and 3a), and geno-serogroup IIb (serotypes 1/2b, 3b, and 7); [d]: P, persistent ; NP, non-persistent

Stock cultures were kept in tryptic soya broth with yeast extract 0.6% (w/v) (TSBYE, Lab M, Heywood, Lanchashire, UK) supplemented with 30% (w/v) of glycerol at –80 °C. Before use, frozen stocks were streaked onto tryptic soya agar with yeast extract 0.6% w/v (TSAYE, Lab M) and incubated at 37 °C overnight. A single colony was inoculated into 10 ml of TSBYE and incubated overnight at 37 ºC. The cultures were then sub-cultured in 10 ml of TSBYE (1% v/v) and incubated at the same conditions. The optical density (OD) of each cell suspension was further measured at 600 nm, and inoculum was prepared by dilution in TSB to obtain approximately 10^6 cells per ml.

1.2. Preparation of stainless steel, silicon rubber and PVC coupons

Stainless steel (type 304) and silicon rubber coupons (2.4 cm×7.3 cm) were washed with distilled water and immersed for 3 min in acetone, followed by a second wash with distilled water and immersed for 3 min in ethanol 70% (v/v). Coupons were then rinsed with distilled water, air-dried, and sterilized at 121 ºC for 15 min. PVC coupons (2.4 cm×7.3 cm) were washed with distilled water, air-dried, and ethylene oxide-sterilized. The sterile coupons were then immersed vertically in 50 ml sterile Falcon tubes (Sarstedt, Nümbrecht, Germany) previously filled with 45 ml of sterile TSB (LabM). All coupons were fitted into Falcon tubes so that both sides were available for bacterial adherence.

1.3. Evaluation of biofilm forming ability in stainless steel, silicon rubber, and PVC coupons by cell enumeration

Three sets of three tubes were set up with each surface material to be tested (i.e. stainless steel, silicon rubber, or PVC), each containing a single coupon, and inoculated with the different strains to achieve a test suspension with approximately 10^6 cells per ml. Tubes were submitted to static incubation for 5 days at 22 ºC; the growth medium was selected based on previous studies that showed acceptable results for biofilm formation by *L. monocytogenes* using TSB (Harvey et al., 2007; Latorre et al., 2011), and temperature of 22 ºC was chosen to represent room temperature. The coupons were then rinsed twice with sterile distilled water and transferred aseptically into a new tube containing 10 ml of sterile phosphate buffer saline (PBS). Biofilms were removed by swabbing on both sides followed by vortexing for 1 min. Serial decimal dilutions were prepared in PBS and inoculated in sterile Petri dish with TSAYE by the drop technique (according to Miles & Misra, 1938) in duplicate. Plates were incubated overnight at 37 °C, and colony enumeration was performed. Two independent experiments were conducted for each surface material (each one with a set of three tubes). *Pseudomonas aeruginosa* ATCC 10145 was used in each assay as a positive control (strong biofilm former; Mohsen et al., 2015). A negative control (tubes with coupons and media without bacteria) was also included. Biofilm formation was determined by calculation of Log_{10} of colony-forming units (CFU) per cm^2.

1.4. Quantification of biofilm by microtiter plate assay

Evaluation of the ability of each strain to form biofilm by microtiter plate assay was performed using a 96 wells sterile polystyrene flat bottom microplate (Orange Scientific, Braine-l'Alleud, Belgium) according to Christensen and co-workers (1985), with minor modifications. Briefly, for each strain six wells were filled with 200 µl of inoculum with approximately 10^6 cells per ml. Six wells with sterile TSB were included as controls. Following static incubation at 22 ºC for 5 days, biofilm formation was assessed by crystal violet staining (Merck, Darmstadt,

Germany). Growth media was removed and wells were gently washed three times with 250 µl of sterile distilled water to remove unattached cells. Attached cells were further fixed using 250 µl of methanol solution (Merck) per well for 15 min and left to air-dry. Crystal violet was added [50 µl of a 0.1% (v/v) solution] to each well and allowed to stain for 45 min at room temperature. Excess stain was removed by gently washing the microplate under running tap water. Microplates were air-dried and 250 µl of a 33% (v/v) acetic acid solution (Merck) was added to each well and incubated for 30 min at 4 ºC. Subsequently, 100 µl from each well were transferred into a new microplate and optical density (OD) was read at 595 nm using a microplate reader (Model 680, Bio-Rad, Hercules, CA, USA). Two independent experiments were performed; for each strain OD values were averaged.

1.5. Statistical analysis

Listeria monocytogenes strains were categorized into two groups: persistent strains and non-persistent strains. Aiming at concluding about significant difference in relation to strains in biofilm formation ability (Log_{10} CFU cm^{-2} and OD_{595}), a Student *t*-test was performed for comparison of means assuming independent samples. Normality and homoscedasticity was assessed for all groups using Kolmogorov–Smirnov and Levene's tests, respectively. The significance level assumed was 5% in all situations. Analyses were performed using IBM SPSS® Statistics® 20 for Windows® (SPSS Inc., Chicago, USA).

2. Results and discussion

Values of Log_{10} CFU cm^{-2} obtained for persistent (n=6) and non-persistent (n=7) strains for each material and microtiter plate assay for the 13 strains are presented in Table 2 and Figure 1. *P. aeruginosa* ATCC 10145 presented the highest values on all tested surfaces. Significant differences in biofilm formation were found between persistent and non-persistent strains on two of the surfaces tested; overall, persistent strains were better biofilm formers on stainless steel (P=0.018) and on silicon rubber (P=0.025). In PVC coupons no relation between persistence and higher ability to form biofilm was found (P=0.141). Overall, the quantity of biofilm produced by each strain was similar on stainless steel and silicon rubber coupons (P=0.914) but different on PVC (P<0.001); the majority of the strains presented higher Log_{10} CFU cm^{-2} value in the latter. Using the microtiter plate assay, no significant difference in biofilm formation was found between persistent and non-persistent strains (P=0.059), and no significant relationship was found with results obtained using the three other surfaces.

Several authors have compared biofilm formation between persistent and non-persistent strains of *L. monocytogenes* isolated from food-contact environments, generating diverse and conflicting results. To our knowledge, nine studies on the subject have been reported so far, all of them using a single methodology: in one study a polystyrene microtiter assay was used (Harvey et al., 2007), in two studies stainless steel coupons were used (Norwood & Gilmour, 1999; Lundén et al., 2000), while in the remaining five a PVC microtiter assay was selected (Djordjevic et al., 2002; Borucki et al., 2003; Jensen et al., 2007; Cruz & Fletcher, 2011; Latorre et al., 2011; Wang et al., 2015). The culture media, growth conditions, time to allow biofilm formation, and temperatures were diverse. Similarly to our results, Norwood and Gilmour (1999) found a statistically significant relationship between persistence of *L. monocytogenes* and ability to form biofilm on stainless steel coupons, as well as Borucki and co-workers (2003) and Latorre and co-workers (2011) using PVC microtiter assays. Harvey

and co-workers (2007) using, like in our study, a polystyrene microtiter assay, found no significant differences in biofilm formation among persistent and non-persistent strains; the same conclusion was drawn by the other studies (Lundén et al., 2000; Djordjevic et al., 2002; Jensen et al., 2007; Cruz & Fletcher, 2011; Wang et al., 2015). It is very important to keep in mind that conclusions drawn from the different studies are always deeply correlated not only with the experimental conditions and strains, as detailed above, but also with the concept of "persistence" itself, as the criteria used to identify persistent and non-persistent strains is not consistent among studies.

Table 2. Biofilm formation for persistent (P) and non-persistent (NP) *L. monocytogenes* isolates and *P. aeruginosa* on abiotic surfaces tested and in the microtiter plate assay

P/NP	Strain	Log_{10} CFU cm^{-2} (Mean±SD)		
		Stainless steel	Silicone	PVC
P	798	3.52±0.21	2.90±0.43	3.82±0.51
	1108	3.27±0.40	3.43±0.24	3.94±0.41
	1597	3.38±0.21	3.88±0.56	3.69±0.32
	1606	3.58±0.17	3.48±0.11	3.60±0.56
	1728	4.22±0.39	3.65±0.37	3.92±0.34
	1757	3.61±0.15	3.75±0.20	3.53±0.32
NP	747	3.68±0.23	3.52±0.14	3.85±0.17
	929	3.64±0.16	3.49±0.32	3.95±0.49
	930	3.16±0.45	3.52±0.32	3.97±0.63
	994	3.03±0.48	3.06±0.34	4.41±0.41
	1302	2.80±0.08	3.16±0.45	3.11±0.24
	1499	3.40±0.46	3.03±0.40	4.38±0.61
	1712	3.48±0.51	3.32±0.34	3,77±0.15
	P. aeruginosa	6.31±0.12	6.58±0.04	6.47±0.13

P: Persistent; NP: non-persistent

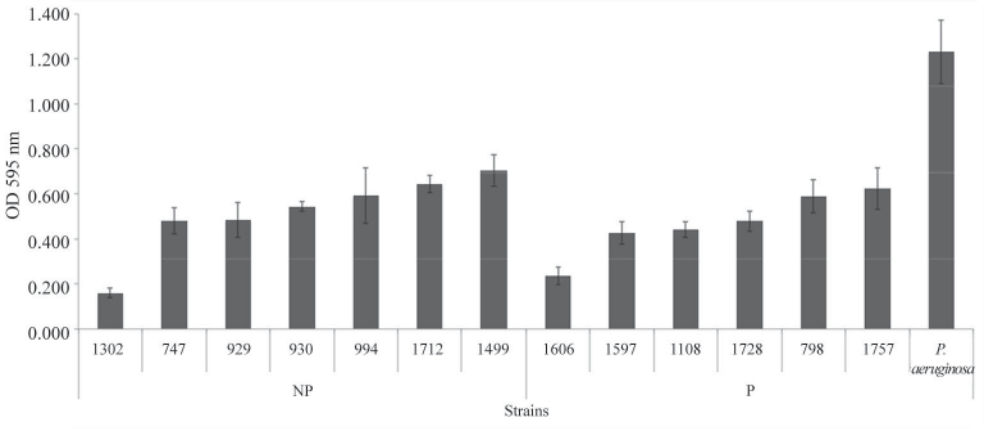

Fig. 1. Values of absorbance at 595 nm obtained for persistent (P) and non-persistent (NP) *L. monocytogenes* isolates and *P. aeruginosa* for biofilm formation incubated at 22 ºC for 5 days

The microtiter plate assay has been widely used to screen differences in biofilm formation among different species, including *L. monocytogenes* isolates. Although it offers significant advantages such as small sample volumes, simultaneous analysis of multiple strains, and rapid turnaround, when compared to other cost- and labour-intensive techniques, drawing inferences from results obtained to estimate the behaviour of *L. monocytogenes* in the surfaces encountered in the processing environment should be taken carefully.

Despite the statistically significant differences obtained in this study, the mean values obtained for persistent and non-persistent strains are quite similar, and a high variability among strains was observed; therefore, the biological significance of these results is unclear. The development of a realistic methodology reflecting the true ability of *L. monocytogenes* to form biofilm in food processing environments is virtually impossible, due to the complexity and diversity of factors involved; however, it would be valuable to establish a standard reproducible method for biofilm quantification among different strains to avoid experimental variables among different studies, other than strain inter-specificity.

3. Conclusions

In this study, biofilm formation by *L. monocytogenes* was dependent on surface materials. Overall, strains tested presented higher ability to form biofilm on PVC, followed by stainless steel and silicon rubber; the number of adhered cells was not significantly different between these two last materials. No correlation was identified between biofilm formation using a microtiter plate assay with crystal violet staining and on the three other surfaces tested. Persistent strains were better biofilm formers on stainless steel and on silicon rubber, while on PVC and in the microtiter assay no relation between persistence and higher ability to form biofilms was found.

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