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Listeria innocua and *Listeria monocytogenes* strains from dairy plants behave similarly in biofilm sanitizer testing

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ABSTRACT

Five *L. innocua* and five *L. monocytogenes*, including persistent and non-persistent isolates collected from Gorgonzola processing plants, were compared regarding their biofilm-forming ability and their biofilm susceptibility to two hydrogen peroxide (HP) based disinfectants in use at the plants. No significant difference in biofilm-forming ability by both species was observed (P>0.05) in crystal violet staining and viable count assays. The susceptibility to HP disinfectants of the *L. monocytogenes* and *L. innocua* biofilms was determined. In order to mimic clean and soiled biofilm forming conditions, biofilms were grown, respectively, in 1/10 diluted TSB-YE and in TSB-YE. The results showed no significant differences between species or conditions (P>0.05) regardless of whether the isolates were classified as persistent or non-persistenttb. A hierarchical clustering based on Principal Component Analysis performed on the tested variables, indicated the presence of two major clusters. Persistent and non-persistent isolates from both species were allocated in both clusters, suggesting that they behaved in a similar way in response to the tested conditions. This study showed that biofilms of in-house *L. innocua* could monitor the effectiveness of HP-based disinfectants. Moreover, biofilms of *L. innocua* could be used as surrogates of *L. monocytogenes* in sanitizer-based biofilm eradication trials simulating dairy processing environments, whenever the use of the pathogen is not an option.

KEYWORDS

Listeria innocua; *Listeria monocytogenes*; biofilms; disinfectants; surrogate.

1. INTRODUCTION

Listeria innocua and *Listeria monocytogenes* have been isolated from the same foods (Lappi et al., 2004; Moshtaghi & Mohamadpour, 2007; Simmons et al., 2014; Vongkamjan, Fuangpaiboon, Turner, & Vuddhakul, 2016) and food processing environments (Chambel et al., 2007; Lappi et al., 2004; Nucera, Morra, & Grassi, 2011; Rørvik, Caugant, & Yndestad, 1995). Sauders et al. (2012) found that both species were associated with urban environments, in contrast with *L. seeligeri* and *L. welshimeri* that were associated with natural environments. Also phylogenetic analysis showed that *L. innocua* and *L. monocytogenes* are closely related species (den Bakker et al., 2010; Glaser et al., 2001; Schmid et al., 2005).

Reviews on *L. monocytogenes* sanitizer susceptibility and persistence have pointed out the difficulty to extrapolate from laboratory-based results to a food processing environment and the need to better understand the involved mechanisms (Carpentier & Cerf, 2011; Ferreira, Wiedmann, Teixeira, & Stasiewicz, 2014). Since *L. innocua* is nonpathogenic, it might be possible to employ this species directly in trials in processing plant environments to investigate its persistence and sanitizer susceptibility rendering valuable data to predict *L. monocytogenes* behavior. In fact, *L. innocua* has already been proposed as a surrogate, although not in biofilm state, in order to predict the response of *L. monocytogenes* to chemical and physical stresses (Delaquis, Stanich, & Toivonen, 2005; Fairchild & Foegeding, 1993; Friedly et al., 2008; Silva-Angulo et al., 2015).

L. monocytogenes is a concern in the production of Gorgonzola cheese, an Italian blue-veined cheese made of pasteurized cow's milk. Some *L. monocytogenes* strains can persist in Gorgonzola processing plants, suggesting niche adaptation to the dairy environment (Lomonaco et al., 2009). The reasons for persistence are not known, but strong biofilm forming ability and disinfectant susceptibility do not seem to be prerequisites (Costa, Bertolotti, Brito, & Civera, 2016). The present study aimed to investigate whether *L. innocua* biofilms may be used as surrogates of the pathogenic *L. monocytogenes* biofilms. Persistent and non-persistent *L. innocua* and *L. monocytogenes* isolates

from Gorgonzola processing environments were selected and their biofilm-forming ability as well as biofilm susceptibility to two in use hydrogen peroxide (HP) based disinfectants were compared.

2. MATERIALS AND METHODS

2.1 Bacterial isolates

The strains used in this work, five *L. innocua* and five *L. monocytogenes*, were isolated from Gorgonzola cheese processing plants and the farms supplying milk, located in Piedmont and Lombardy, Italy (Table1). Isolates were classified as persistent if they were found repeatedly within the collection periods indicated in Table 1, and were at least 95% similar according to repetitive element sequence-based PCR assays (rep-PCRs) (Nucera et al., 2011; Nucera, Lomonaco, Costa, Morra, & Grassi, 2013) using ERIC and REP primers (Versalovic, Koeuth, & Lupski, 1991)

2.2 Evaluation of biofilm-forming ability and biofilm susceptibility

The evaluation of biofilm-forming ability was performed by quantification of biofilm biomass on microtiter plates (polystyrene), using both the crystal violet (CV) method (Borucki, Peppin, White, Loge, & Call, 2003) and a method of enumeration of viable cells on stainless steel coupons (SSC), as described by Costa et al. (2016). Biofilms were grown in tryptic soy broth with 0.6% yeast extract (TSB-YE, Biokar Diagnostics, Beauvais, France) at 25 °C for 24 (CV) and 48 h (SSC). The CV approach was replicated at least three times on biologically independent cultures on distinct days (biological replicates), with six repetitions under identical conditions each (technical replicates). For the cell enumeration, two biological replicates were performed, with two technical replicates each.

The evaluation of the biofilm susceptibility to the disinfectants was performed on biofilms grown on SCC in nutrient-limiting (1/10 diluted TSB-YE) and in nutrient-rich (TSB-YE) medium at 25 °C for 48 h, as described by Costa et al. (2016). Two commercial HP-based disinfectants, commonly employed at the dairies where the isolates were collected, were used: P3-oxonia active (ECOLAB S.r.l) containing acetic acid and peracetic acid (designated herein as P3), and Mida San 315

(Christeyns Food Hygiene S.r.l) containing citric acid (designated herein as MS). Disinfectants were diluted using sterile hard water, prepared according to EN 13697 (Anonymous, 2001), to achieve the concentrations recommended by the manufacturers: 0.2% (v/v) and 0.5% (v/v) for P3, and 0.5% (v/v) and 1% (v/v) for MS. Disinfectant efficiency was defined according to EN 13697 (Anonymous, 2001), that states that a minimum 4-log reduction in viable cells is required. If that threshold was obtained in 2.5 min with the lowest concentration, no additional treatments were performed. When a 4-log reduction was not achieved, 5- and 7.5-min treatments with higher disinfectant concentrations, when needed, were carried out until a 4-log reduction was observed. For each isolate, a control was exposed to sterile hard water and used for the calculation of the log reduction. Each treatment was repeated under identical conditions on two distinct biofilms.

2.3 Data analysis

Agreement to a normal distribution of the data generated by the CV (A_{600}), enumeration on SSC (Log CFU/cm^2), and biofilm susceptibility (reduction of log CFU/cm^2) assays was checked using the Shapiro-Wilk test, and the homogeneity of the variance was confirmed by Levene's test. Comparisons between means were then performed via one-way ANOVA, using Scheffé test. These analyses were performed with the OriginPro 8 SR0 (Northampton, MA, USA) software.

Using JMP Pro 13.2.1 (SAS, Cary, NC USA) software, a principal component analysis (PCA) was performed in order to compare the isolates, based on their response to the ten following conditions of log CFU/cm^2 reduction on SSC after disinfectant (P3 or MS) treatment of biofilms grown in clean (C) and soiled (S) conditions, and of biofilm formation: 1) P3 2.5 min 0.2% C; 2) P3 2.5 min 0.2% S; 3) P3 5 min 0.2% C; 4) P3 5 min 0.2% S; 5) MS 2.5 min 0.5% C; 6) MS 2.5 min 0.5% S; 7) MS 5 min 0.5% C; 8) MS 5 min 0.5% S; 9) biofilm formation by CV; and 10) biofilm formation by enumeration on SSC. A hierarchical clustering using the Ward method was performed based on the component 1, which explained the majority of the variance.

For all tests, the confidence level for significance was 95 % ($P<0.05$).

3. RESULTS

3.1 Comparison of the biofilm-forming ability of the isolates

The biofilm formation of *L. innocua* isolates ranged from 0.117 and 0.170 (A_{600}) using the CV method while the enumeration method registered values between 6.20 and 6.69 log CFU/cm^2 (Fig. 1). When *L. innocua* isolates biofilm-formation values are presented alongside *L. monocytogenes* values (Costa et al., 2016), it is possible to observe that *L. innocua* presented a similar range for both the CV method (0.087-0.270) and enumeration method (5.65-6.74 log CFU/cm^2) (Fig.1).

The CV assay relies on the property of the dye to bind to negatively charged surface molecules and polysaccharides in the matrix. This assay may be influenced by the amount of exocellular polymer and by cell sedimentation, which increases with planktonic growth (Lourenco, Rego, Brito, & Frank, 2012). This may explain data variability obtain with this method. However, the CV method showed no significant differences between isolates nor between species ($P>0.05$, Fig. 1B). The same outcome was obtained for the enumeration on SSC, except for *L. monocytogenes* isolate GR, that was significantly different from *L. monocytogenes* isolates GI and GN and from *L. innocua* isolate 2, with P-values of 0.011, 0.009 and 0.016, respectively (Fig. 1A).

3.2 Comparison of the antibiofilm activity of the disinfectants

The log reductions obtained after the treatment of *L. innocua* biofilms (light dots) with the disinfectants P3 and MS are shown in Fig. 2. In order to allow comparison between species, data from *L. monocytogenes* isolates (dark dots) (Costa et al., 2016) are also shown. Among the isolates that did not reach the 4-log reduction threshold, and for that reason were consequently exposed to longer treatments and/or higher concentrations of P3 and MS, *L. monocytogenes* and *L. innocua* persistent and non-persistent isolates were found. This was observed when biofilms were grown both in nutrient-limiting (1/10 diluted TSB-YE) and in nutrient-rich (TSB-YE) conditions (Fig. 2A-I, quadrants II, III and IV). Moreover, when the log reduction values obtained with both disinfectants at all the tested concentrations and contact times were compared, there were no

significant differences between isolates nor between species (P>0.05). The exception was the comparison of susceptibility to P3 at the mildest exposure conditions (0.2% [v/v] for 2.5 min) in which *L. monocytogenes* presented a higher log reduction (P-value of 0.0497).

The disinfectant P3 showed a greater efficacy than MS at 7.5 minutes of exposure and at the lowest concentration indicated by the manufacturer (0.2% [v/v]), P3 was effective for all the isolates Fig. 2A-C) and the treatment with the higher concentration (0.5% [v/v]) was not required. For the same exposure time, the lowest concentration of MS (0.5% [v/v]) was not enough to achieve a 4-log reduction by all the isolates (Fig. 2D-F) and a higher concentration (1% [v/v]) was needed (Fig. 2G-I).

3.3 Comparison of *L. monocytogenes* and *L. innocua* by PCA

Principal Component Analysis (PCA) of data from the 10 isolates regarding biofilm forming ability (CV and SSC) and disinfectant (P3 and MS) susceptibility was performed. Data were from biofilms produced in nutrient-limiting (clean) (1/10 diluted TSB-YE) and in nutrient-rich (soiled) (TSB-YE) conditions. The log reduction values of the 10 isolates exposed to the mildest disinfectant concentrations (0.2% for P3 and 0.5% for MS) for 2.5 and 5 minutes were considered (Fig. 2A, B, D and E). These were the treatments applied to the 10 isolates, since isolates that after these treatments reached the 4-log reduction threshold were not subsequently exposed.

By PCA, the initial 10-dimensional space (10 variables) was reduced to a plane F1F2, defined by the two first principal components. This plane accounts for about 68.5% of the variance of the original data (Fig. 3). The projection of the 10 original variables on the first two principal components is presented in Fig. 3A. Except for the treatment with P3 for 5 min at 0.2% in both clean and soiled conditions, in general, disinfectant susceptibility (log reduction) shows a positive correlation with the first component increasing along it. The second principal component is positively correlated with both CV and SSC values, i.e. biofilm production increase along this axis.

The projection of the different isolates in the plane F1F2 is presented in Fig. 3B. The isolates in quadrant I (2, GN, GI and 4) and in quadrant II (1) are more susceptible to P3 as all of them reached the 4-log reduction threshold after exposure for 5 min to 0.2% (v/v) (Fig. 3, and Fig. 2B). Isolates in quadrants III (99, 5, 3 and G39) and IV (GR) were less susceptible to P3. These isolates only achieved the 4-log reduction after a 7.5 min exposure to the same concentration (Fig. 3, and Fig. 2C). Isolate GR can be seen isolated on quadrant IV as it presented the higher log reductions in both clean and soiled condition for the eight treatments used to perform the PCA (Fig 3, and Fig. 2A, B, D and E). Moreover, isolate GR is worst biofilm producer than three (2, GN and GI) of the four isolates positioned in quadrant II (Fig. 3 and Fig. 1).

A hierarchical clustering, based on the first principal component which explains the majority (43.9%) of the variance (Fig. 3) was performed and allowed to confirm the presence of the three clusters of isolates (C1, C2 and C3) (Fig. 4), as suggested by PCA. A two-way clustering was performed. According to the intensity of the response of each isolate in every experiment, the treatments with P3 for 5 min at 0.2%, in both clean and soiled conditions, were the ones that allowed the most differentiation of the isolates. It is also possible to observe that, persistent and non-persistent isolates from both species *L. innocua* and *L. monocytogenes* were allocated in both clusters, confirming that both species respond in a similar way to the conditions tested in this work.

4. DISCUSSION

The use of *Listeria* spp. as an indicator of a possible contamination by *L. monocytogenes* has been suggested by some authors and guidelines (Food and Drug Administration [FDA], 2008; Pennsylvania State University [Penn State], 2003; Tompkin, Scott, Bernard, Sveum, & Gombas, 1999). This may be a conservative approach since in Gorgonzola processing plant *L. innocua* was far more frequent than *L. monocytogenes* (Nucera et al., 2001). In fact, repeated positive testing for *Listeria* spp. requires more stringent cleaning and disinfecting procedures and indicates the need to elucidate the reasons for these positive results.

Meylheuc, Giovannacci, Briandet, and Bellon-Fontaine (2002) have compared the bioadhesive behavior of both species and concluded that the non-pathogenic strain exhibited a more marked electronegative character and a slightly more hydrophilic nature than *L. monocytogenes*. Nevertheless, to our knowledge, only two studies have previously compared the biofilm production of *L. monocytogenes* and *L. innocua*: Zhou et al. (2011) used the CV method and concluded that *L. innocua* is a weaker biofilm former compared to *L. monocytogene*s; Koo, Ndahetuye, O'Bryan, Ricke, and Crandall (2014) used the cell enumeration method on aluminum and stainless steel and concluded that after 24 h the attachment of *L. monocytogenes* was significantly higher than that of *L. innocua*, though no significant differences were observed between both species biofilms after 72 h. In the present work, two methods were used (CV in polystyrene P96 microtiter plates and enumeration on SSC) to evaluate the biofilm forming ability of a set of *L. innocua* and *L. monocytogenes* isolates fairly representative of the contaminant microorganisms, collected from Gorgonzola processing plants. The comparison performed here, indicated that both species produced similar values, suggesting an equivalent biofilm production.

Regarding disinfectant susceptibility, Best, Kennedy, and Coates (1990) tested the efficacy of 14 disinfectants against both species after been spotted onto the surface of stainless steel disks and dried for 30 minutes. The obtained results showed that the pathogenic species was slightly less susceptible to disinfection than *L. innocua*. A few other comparative studies have assessed differences in the susceptibility of planktonic cells of both species to disinfectants finding no differences between species (Margolles, Mayo & de los Reyes-Gavilán, 2000) or highlighting an higher resistance of planktonic *L. innocua* (Yeater, Kirsch, Taylor, Mitchell, & Osburn, 2015).

In this work, using *L. innocua* and *L. monocytogenes* collected from the same environment, a similar susceptibility to the tested disinfectants was found for biofilms of both species. Moreover, the susceptibility to P3 and to MS of biofilms grown in conditions mimicking clean and soiled environment, showed no significant differences in terms of log reduction between persistent and non-persistent isolates. According to these results, *L. innocua* could be used as a surrogate for *L.*

monocytogenes, not only regarding the biofilm production, but also the biofilm susceptibility to HP-based disinfectants.

L. innocua was the only *Listeria* species besides *L. monocytogenes* detected in the Gorgonzola processing plants from where the isolates analyzed in this work were collected (Nucera et al., 2011). Our results demonstrate that the presence of *L. innocua* could indicate a contamination by *L. monocytogenes*, since the disinfectant susceptibility of the two species was similar. In fact, as concluded in the review by Milillo et al. (2012), if the two species have adapted to fit different environmental niches, they may not always respond to stress the same way. Therefore, the absence of *Listeria* spp. (i.e. *L. innocua* and *L. monocytogenes*) on food contact surfaces, equipment and floors would suggest the effectiveness of the sanitation procedures in place in the processing plant. Conversely, the detection of positive samples for *Listeria* spp. would indicate a need of improving the procedures to keep the environmental contamination under control, as suggested by Tompkin et al. (1999). Moreover, Zitz, Zunabovic, Domig, Wilrich, and Kneifel (2011) also verified reduced detectability of *L. monocytogenes* in the presence of *L. innocua* mainly due to the overgrowth of *L. monocytogenes* by *L. innocua* during the selective enrichment, leading to false-negative results. Furthermore, the use of *Listeria* spp. as an indicator of a potential *L. monocytogenes* contamination represents lower costs for routine laboratory analysis, due to the higher cost of chromogenic media used for *L. monocytogenes* (Tomkin, 2002).

The presented work tried to mimic food industry conditions. Consequently, susceptibility testing was conducted with biofilms produced on stainless steel, in soiled and in clean conditions, and testing two commonly used HP-based disinfectants at the dairy plants where the isolates were collected from. Nevertheless, in the real industrial environment the strains will form multi-species biofilms and that may become relevant to the response of both *L. monocytogenes* and *L. innocua* to disinfectants.

4. CONCLUSION

This study has shown that biofilms of in-house *L. innocua* could be employed for the validation and monitoring of HP-based disinfectant efficacy and proper sanitation procedures in Gorgonzola processing plants. In fact, not only *L. innocua s*usceptibility to HP-based disinfectants is similar to *L. monocytogenes* biofilms, but also both species, collected from the same food industry environment, showed no differences in biofilm forming ability. The common origin of the isolates is probably fundamental when looking for the adequacy of *L. innocua* as a surrogate of *L. monocytogenes*. The convenience and safety in using a non-pathogenic surrogate will certainly contribute to clarify the factors that contribute to *L. monocytogenes* persistent colonization not only in some Gorgonzola processing plants as in other food industry environments.

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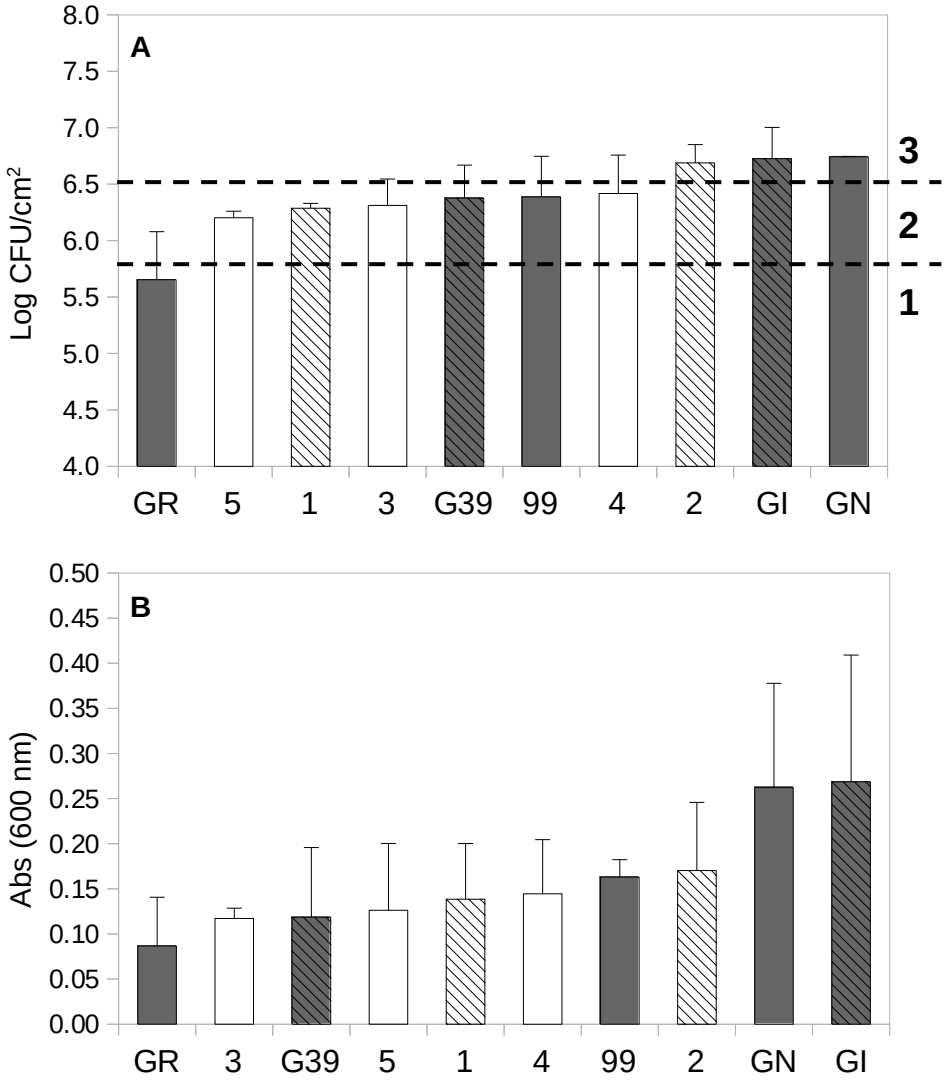

Fig. 1 Biofilm forming ability of *Listeria monocytogenes* (grey bars) and *Listeria innocua* (white bars). Oblique line bars represent persistent isolates; A - assessed by cell enumeration on SSC (grown in TSB-YE for 48 h at 25 °C). The isolate GR, only on region 1 is statistically different from isolates 2, GI and GN, on region 3 (regions marked on the right), The isolates on region 2 are neither statistically different from isolates on region 1 nor from region 3. Two biological replicates with two technical replicates each were performed; **B** - assessed by crystal violet (CV) method in polystyrene 96-well microtiter plates (grown in TSB-YE for 24 h at 25 °C). Isolates were not statically different (P> 0.05). *L. innocua* isolates 1 and 2 and *L. monocytogenes* isolates GR and GI are persistent isolates (Table 1). Results from *L. monocytogenes* were previously published by Costa et al. (2016) and are shown here only for comparison. Error bars represent standard deviations. At least three biological replicates were performed, with six technical replicates, each.

Fig. 2 Log reductions (log CFU/cm2) obtained after disinfectant treatment of *L. innocua* (light dots) and *L. monocytogenes* (dark dots) biofilms grown for 48 h at 25 °C on SSC, in clean conditions (1/10 TSB-YE; x-axis) and soiled conditions (TSB-YE; y-axis), using P3 at 0.2% or MS at 0.5% and 1%. The isolates placed in quadrant **III** did not reach the 4-log reduction neither with biofilms formed in soiled conditions nor with biofilms formed in clean conditions and further treatments with extended contact time/increased disinfectant concentration were performed, as needed. The isolates placed in quadrant **I** reached the reduction threshold of 4 logs, with biofilms formed in both conditions and, for this reason, no other treatments were performed. The isolates placed in quadrant **II** and **IV** reached the 4 log reduction threshold, respectively, only in soiled or clean biofilm forming conditions. Further treatments respectively with biofilms grown in clean and soiled conditions, were carried out in order to achieve the 4-log reduction in both conditions. Isolates that underwent treatment only with biofilms produced under clean/soiled condition are marked with an asterisk (*) and placed on x- or y-axis, respectively. Isolates' ID in bold represent persistent isolates. Results from *L. monocytogenes* were previously published by Costa et al. (2016) and are shown here only for comparison. Error bars represent standard deviations. For each treatment, two technical replicates were performed.