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Biofilm formation and disinfectant susceptibility of persistent and non-persistent *Listeria monocytogenes* isolates from Gorgonzola cheese processing plants

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Running title: Persistence of *L. monocytogenes* in Gorgonzola dairies

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ABSTRACT

The aim of this study was to investigate whether the biofilm-forming ability and/or the disinfectant susceptibility accounted for the persistence of *Listeria monocytogenes* in Gorgonzola cheese processing plants. For this purpose, a set of 16 *L. monocytogenes* isolates collected in the 2004-2007 period was analyzed, including 11 persistent isolates collected in different years, within the collection period, and displaying identical or highly correlated pulsotypes. The evaluation of biofilm-forming ability was assessed using crystal violet (CV) staining and the enumeration of viable cells on stainless steel coupons (SCC). Absorbance values obtained with CV staining for persistent and non-persistent isolates were not significantly different (rm-ANOVA $p > 0.05$) and the cell counts from non-persistent isolates showed to be higher than of persistent isolates (rm-ANOVA $p < 0.05$). A simulation of disinfectant treatments was performed on spot inoculated coupons in clean and dirty conditions, according to EN 13697, and on biofilms on SSC, grown in nutrient-rich (dirty) and limiting (clean) conditions using acid acetic-hydrogen peroxide (P3) and acid citric-hydrogen peroxide (MS) commercial disinfectants. The treatment was considered effective when a 4 Log reduction in viable cell count was observed. The Log reductions of persistent and non-persistent isolates, obtained with both the assays in clean and dirty conditions, were compared and no significant differences were detected (rm-ANOVA $p > 0.05$). A greater influence of organic matter on MS could explain why P3 was efficient in reducing to effective levels the majority of the isolates at the lowest concentration suggested by the manufacturer (0.2% [v/v]), while the same purpose required an higher concentration (1% [v/v]) of MS.

In conclusion, our results demonstrate that the persistence of these isolates in Gorgonzola cheese processing plants was linked neither to the biofilm forming ability nor to their susceptibility to hydrogen peroxide-based disinfectants, therefore other factors should contribute to the persistent colonization of the dairies.

Key words: Biofilm, Disinfectant, Gorgonzola, Persistence, Susceptibility

INTRODUCTION

Listeria monocytogenes is the etiological agent of human listeriosis, a foodborne disease that caused, in 2014 in the European Union, 0.52 cases per 100,000 population (EFSA, 2015). Although the low incidence, the impact on public health is high since sepsis, meningoenzephalitis and abortions, can occur in neonates, elderly, immune-compromised people and pregnant women (Doganay, 2003).

Ready-to-eat (RTE) foods can be considered at high risk for the transmission of *L. monocytogenes* since it is able to adapt to food preservation methods and RTE foods can be consumed without heat treatments (Wagner and McLauchlin, 2008). Among RTE foods, dairy products were associated with approximately half of the listeriosis outbreaks in Europe since 1985 (Melo et al., 2015).

Gorgonzola is an Italian blue-veined cheese made of pasteurized cow's milk, frequently contaminated by *L. monocytogenes*. Generally, the contamination is limited to the rind, but it could be transferred to the paste during handling (Bernini et al., 2013; Lomonaco et al., 2009; Manfreda et al., 2005). In 2003, a case of listeriosis associated with the consumption of Gorgonzola, was described. Pulsed-Field Gel Electrophoresis (PFGE) and Random Amplification of Polymorphic DNA (RAPD) subtyping showed that the clinical isolate was indistinguishable from those collected from the processing plant and that closely related isolates persisted for five months in the dairy (Gianfranceschi et al., 2006).

Different subtyping methods proved that specific isolates of *L. monocytogenes* are able to establish persistent contaminations in dairy premises for months and sometimes even years (Chambel et al., 2007; Leite et al., 2006; R uckerl et al., 2014). The mechanisms leading to the persistence are poorly understood. Some authors reported that characteristics such as the low susceptibility to disinfectants (Aase et al., 2000; Lund en et al., 2003) and the biofilm formation (Borucki et al., 2003) might facilitate the persistence of certain strains in food processing plants. However, other researchers did not find the persistent strains less susceptible to disinfectants (Heir et al., 2004; Holah et al., 2002)

neither were those strains stronger biofilm producers (Harvey et al., 2007; Nilsson et al., 2011; Ochiai et al., 2014).

Given the complexity of the problem, understanding the reasons for the persistence of *L. monocytogenes* in Gorgonzola processing plants is of utmost importance. The aim of this study was to investigate whether persistent isolates differed from presumed non-persistent isolates in biofilm forming ability and in susceptibility to two disinfectants commonly used in the dairies.

MATERIALS AND METHODS

Bacterial isolates

Lomonaco et al. (2009), during a four-year period (2004 to 2007), collected 95 *L. monocytogenes* isolates from 22 Gorgonzola cheese processing plants. Those isolates were typed by PFGE and 24 displayed 24 different pulsotypes and were considered “uniques”, while the other 71 isolates were grouped into five pulsotypes (A, B, C, D and E). When identical (indistinguishable patterns) or highly correlated (similarity level $\geq 95\%$) pulsotypes were detected in different years within the collection period (2004-2007), the corresponding isolates were considered persistent. These isolates that persisted in the same processing plant or within different dairies suggested, as already reported by others (Leite et al., 2006), niche-adaptation of particular strains in the dairy environment.

In agreement with this, the 16 *L. monocytogenes* isolates analyzed in this work, five non-persistent and 11 persistent (Table 1), were selected from that initial panel of 95 isolates, according to their genetic diversity. Cultures were stored at -20°C in cryoprotectant on cryogenic beads (Cryobank, Merseyside, UK) until use.

Stainless steel coupons

Stainless steel coupons (SSC - 1×1 cm) type 316 finish 4b (University of Georgia instrument shop, Athens) were used. Before use, they were cleaned in acetone, rinsed in distilled water and immersed in a phosphoric-acid-based cleaner (CIP 200, Steris Corp., Mississauga, Ontario, Canada) for 20 min. The coupons were rinsed again and sterilized individually by autoclaving in test tubes.

Evaluation of biofilm-forming ability

The evaluation of biofilm-forming ability of the isolates was performed using (i) the crystal violet (CV) staining method and (ii) the enumeration of viable cells on SSC.

i) CV staining

The CV staining was performed as described by Lourenço et al. (2012). Bacterial colonies, grown overnight at 37°C on tryptic soy agar with 0.6% yeast extract (TSAYE; Biokar Diagnostics, Beauvais, France), were suspended in tryptic soy broth with 0.6% yeast extract (TBSYE; Biokar Diagnostics, Beauvais, France) to achieve a concentration of 1×10^8 CFU/ml ($A_{600}=0.5$). One hundred and fifty μ l of each suspension and of sterile TSBYE were aliquoted in six wells of a polystyrene 96-well microtiter plate (Orange Scientific, Braine-l'Alleud, Belgium). The plate was covered and sealed with Parafilm, to prevent evaporation, and incubated in an orbital shaker at 150 rpm (MIR-154-PE, Panasonic, Japan) for 24 h at 25°C. Biofilms were washed and stained for 45 min with 50 μ l of a 0.1% (w/v) aqueous solution of CV. The absorbed CV was solubilized by adding 200 μ l of 95% (v/v) ethanol and incubating at 4°C for 30 min. From each well, 100 μ l was transferred to a new microtiter plate and the absorbance measured at 600 nm using a microplate reader (model 680, Bio-Rad, Hercules, CA). At least three biological replicates were performed, with six technical replicates, each.

ii) Cell enumeration on SSC

For each culture, one isolated bacterial colony from the surface of TSAYE plate was suspended in 10 ml of TSBYE. Coupons were immediately immersed in 1.5 ml aliquots of these suspensions and

incubated, for 48 h at 25°C, in properly covered and Parafilm sealed 24-well microplates (Orange Scientific, Braine-l'Alleud, Belgium). After the incubation period, coupons were rinsed with one ml of Ringer's solution on both side to remove unattached cells and placed in a new 24-well microplate containing one ml of TSBYE and 20 glass beads (3 mm diameter) below and 30 above the coupon. Then, the microplate was vortexed for one min at maximum speed to detach cells. The amount of beads and vortexing time were previously optimized in order to achieve the maximum viable cell recovery rate. The resulting suspension was transferred to a test tube, decimal diluted and spot inoculated on TSAYE plates. Two biological replicates were performed, with two technical replicates, each.

Disinfectant solutions

Two commercial disinfectants commonly used in the dairies were evaluated. The active ingredients of the first disinfectant, P3-oxonia active, ECOLAB S.r.l. (designated herein as P3), were hydrogen peroxide (HP), acetic acid and peracetic acid, while the second disinfectant, Mida San 315, Christeyns Food Hygiene S.r.l. (designated herein as MS), was composed by HP and citric acid. Disinfectants were diluted in hard water (magnesium chloride, calcium chloride and sodium bicarbonate, pH 7 ± 0.2), prepared according to EN 13697 (Anonymous, 2001), to achieve the concentrations indicated 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.

Evaluation of the listericidal activity of the disinfectants

The evaluation of the listericidal activity of the two disinfectants was performed on (i) spot inoculated SSC and (ii) biofilms on SSC. The exposure times at the concentrations indicated above were established according to preliminary results.

The treatment was considered effective when a 4 Log reduction (difference between Log CFU/cm² values of spotted cells or of biofilms exposed to hard water and the corresponding values obtained after exposure to disinfectant solutions) was observed.

i) Spot inoculated SSC

The listericidal activity of the disinfectants was evaluated using the EN 13697 (Anonymous, 2001) on five (G39, G02, G65, 93, GR) of the 16 isolates (Table 1). These isolates had previously shown, in planktonic state, high minimum bactericidal concentration values for the two disinfectants (0.5% and 1% for P3 and 0.5% and 2% for MS [v/v]). These minimum bactericidal concentration values were obtained by incubating the isolates in brain heart infusion (BHI) supplemented with twofold dilutions of each disinfectant, for 24 h at 37°C, followed by inoculation onto Oxoid Chromogenic Listeria Agar (OCLA) (unpublished data).

Bacterial colonies from the surface of TSAYE plates were suspended in 10 ml of tryptone sodium chloride solution to achieve a concentration of $1.5-5 \times 10^8$ CFU/ml ($A_{600}=0.5$). Two interfering solutions containing 0.3 g/l (clean conditions), or 3 g/l (dirty conditions) of bovine albumin and 1 g/l of tryptone were prepared according to EN 13697, in order to simulate a surface in satisfactory or poor hygienic conditions. One ml of each interfering solution was mixed with one ml of each bacterial suspension (test suspension). The coupons, in a Petri dish, were spot inoculated with 50 μ l of the test suspension (final amount about 10^6 CFU), dried at room temperature and transferred into a 24-well microplate, where 100 μ l of the disinfectant solution (lowest concentration) was placed onto the spot inoculated coupons. The coupons were exposed for 2.5 and 5 min for P3 and MS, respectively, at 25°C, or for 5 min for P3 and 7.5 min for MS, as needed, to achieve the 4 Log reduction threshold. For each isolate, hard water was used as a control. Then, the coupons were transferred into a new 24-well microplate containing one ml of Dey/Engley Neutralizing broth (D/E - Difco, Becton Dickinson, Heidelberg, Germany). Thirty glass beads were placed above the coupon and used to detach the cells by vortexing, as described before. After a 5 min neutralization period, the suspension was decimal diluted and TSAYE plates were inoculated by the pour plate method. For each treatment (disinfectant concentration and contact time), two technical replicates were performed.

ii) Biofilms on SSC

Biofilms were grown in nutrient-limiting (1/10 diluted TSBYE) and in nutrient-rich (TSBYE) conditions, to simulate clean and dirty biofilm forming conditions. The procedure for cell enumeration on SSC was performed as previously described.

After rinsing with one ml of Ringer's solution, coupons were immersed in one ml of each disinfectant solution, beginning with the lowest concentrations for 2.5 min at 25°C. Contact times were extended to 5 and 7.5 min and disinfectant concentrations have been increased as needed, in order to achieve the 4 Log reduction threshold. As before, for each isolate, hard water was used as a control. After disinfectant exposure, the coupons were rinsed and vortexed in one ml of D/E with glass beads in order to detach adhered cells, as described before. After a 5 min neutralization period, one ml of the undiluted suspension was inoculated in TSAYE plates by the pour plate method; the remaining suspension was decimal diluted and 100 µl of each dilution was spread onto TSAYE plates. For each treatment (disinfectant concentration and contact time), two technical replicates were performed.

Data analysis

Statistical analyses were performed by using the R statistical software (R Core Team 2012). For all tests, the confidence level for significance was 95% ($p < 0.05$). Differences in terms of biofilm production and Log reduction among persistent and non-persistent isolates and clean and dirty conditions were evaluated by ANOVA for repeated measures (rm-ANOVA), after the evaluation of normal distribution of the data (Shapiro Wilk test).

RESULTS

Evaluation of biofilm-forming ability

The biofilm-forming ability of the 16 isolates evaluated by the CV staining method ranged from about 0.04 (G46) to 0.30 (GH) (A_{600}), whereas the population of viable attached cells on SSC ranged from 5.13 (G74) to 6.80 (GM) Log CFU/cm² (Fig. 1).

According to the values obtained, the isolates were arbitrarily classified as weak, moderate or strong biofilm producers (Fig. 1). Weak producers, according to CV staining, corresponded to moderate producers identified by the cell enumeration method. Furthermore, the group classified as strong producers by this last method includes moderate and strong producers classified by the CV staining (Fig. 1).

When the CV results were analyzed, there was no significant difference (rm-ANOVA $p>0.05$) between persistent and non-persistent isolates (Fig. 1). However, with cell enumeration method a significant difference between persistent and non-persistent biofilm values was observed (rm-ANOVA $p<0.05$), suggesting a possible greater attachment for non-persistent isolates (Fig. 1).

Evaluation of the listericidal activity of the disinfectants

Disinfectants P3 and MS were tested at concentrations indicated by the manufacturer: for P3, 0.2 and 0.5% (v/v), and for MS 0.5 and 1% (v/v), for contact times varying from 2.5 to 7.5 minutes, as needed to achieve the 4 Log reduction.

i) Spot inoculated SSC

Observing the results of the disinfection treatments of spot inoculated SSC, the lowest concentration of P3 indicated by the manufacturer (0.2% [v/v]) was effective for the inactivation of all the isolates (Fig. 2A); contrarily the lowest concentration of MS (0.5% [v/v]) was not effective and a higher concentration (1% [v/v]) was needed for the inactivation of 4/5 isolates (G02, G39, GR, 93) in clean conditions, and of all the isolates in dirty conditions (Fig. 2B).

There was no significant difference between persistent and non-persistent isolates in terms of Log reduction for both disinfectants and they displayed similar susceptibility under both clean and dirty conditions (rm-ANOVA $p>0.05$, Fig. 2). However for P3 in dirty conditions a higher number of isolates ($n=4$) was exposed to prolonged treatments than in clean conditions and, regarding MS, in dirty conditions, two isolates (93, G65) required a treatment of 7.5 min (Fig. 2).

i) Biofilms on SSC

The lowest concentration of P3 (0.2% [v/v]) was able to provide a 4 Log reduction in 13/16 isolates (Fig. 3) while for MS a concentration of 1% (v/v) was necessary for the inactivation of 12/16 isolates in clean conditions and 13/16 in dirty conditions (Fig. 4).

No significant differences between persistent and non-persistence isolates in terms of Log reduction were detected for both disinfectants under nutrient-limiting and nutrient-rich conditions (rm-ANOVA $p > 0.05$, Fig. 3 and Fig. 4).

DISCUSSION

In this study, our main goal was to determine if the biofilm-forming ability and the susceptibility to two HP-based disinfectants could be considered as possible cause of the persistence of *L. monocytogenes* isolates from Gorgonzola cheese processing plants.

All the isolates were able to form biofilms and a variability in biofilm production among isolates was observed, as previously reported (Borucki et al., 2003; Harvey et al., 2007; Kadam et al., 2013; Nilsson et al., 2011). Results obtained with CV staining, which estimates the total cells and polysaccharides in the matrix are in accordance with the viable cell enumeration method results. In fact, the number of strong biofilm producers identified by the CV staining method was 4/16, and with the cell enumeration on SSC this number was 7/16 isolates. In accordance, four isolates (GM, GN, GI, GH) were identified as strong biofilm producers by both approaches. Therefore, we could assume that these biofilms are characterized by an high cellular density, rather than an high content in polysaccharides in the matrix, as already described by others using different methods (Nilsson et al., 2011; Renier et al., 2011).

In this work, absorbance values obtained with CV staining method for persistent and non-persistent isolates were not significantly different. Despite this, the cell counts in SSC from non-persistent isolates showed to be higher than those of persistent isolates. Although this result might be affected

by the different number of persistent (11) and non-persistent (five) isolates analyzed here, Nilsson et al. (2011) also showed that biofilms of non-persistent isolates seem to include more cells than biofilms of persistent isolates. This suggests that persistence of *L. monocytogenes* in Gorgonzola dairies may be not related to the biofilm-forming ability of the contaminant strains, in contrast to what was stated by others (Borucki et al., 2003; Lundén et al., 2000). All the same, other factors, related to surface properties, surrounding bulk fluid, cell characteristics and/or presence of other bacterial species may affect cell adhesion and/or biofilm formation. All of these factors may determine the persistence of certain strains in a given environment (Simões et al., 2010).

Aiming to determine if disinfectant susceptibility accounted for persistence, the simulation of disinfectant treatments was performed using target cells in two forms: spot inoculated SSC (EN 13697), and biofilms grown on SSC in rich or in nutrient-limiting conditions. When the Log reductions obtained with both the assays were compared, no significant differences were detected between persistent and non persistent isolates, suggesting, in agreement with previous studies (Cruz and Fletcher, 2012; Kastbjerg and Gram, 2009), that persistence does not seem to be related with disinfectant susceptibility.

While the listericidal effect of the combination of HP and acetic acid is well established, at our knowledge, the susceptibility of *L. monocytogenes* to an acid citric-HP-based commercial disinfectant has never been assessed by others. Martin and Maris (2012) demonstrated a synergistic effect of the association of HP with acetic acid against a *L. monocytogenes* strain, however the combination of HP with citric acid not only did not show any synergistic effect but was also affected by mineral and organic interfering substances. Our results seem to be consistent with these findings, since a greater influence of organic matter on MS (HP and citric acid) could explain why P3 (HP and acetic acid) was efficient in reducing to effective levels the majority of the isolates at the lowest concentration suggested by the manufacturer (0.2% [v/v]), while the same purpose required an higher concentration (1% [v/v]) of MS.

CONCLUSIONS

Our results demonstrate that the persistence of these isolates in Gorgonzola cheese processing plants was not linked either to the biofilm forming ability of the isolates nor to their susceptibility to HP-based disinfectants, therefore other factors should contribute to the persistent colonization of the dairies. Carpentier and Cerf (2011) concluded that there are no strains with unique properties leading to persistence in food premises and that the cause may be our inability to kill or to dislodge them from harborage sites. Moreover, the in-house microbiota may influence the adhesion of *L. monocytogenes* to surfaces since it has been proved that some species can enhance or inhibit *L. monocytogenes* colonization (Carpentier and Chassaing, 2004). Thus, future investigations may analyze the role of the resident microbiota in the persistence of *L. monocytogenes* in cheese processing plants.

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Table 1 *Listeria monocytogenes* isolates used in this study

Isolate ID	Processing plant	Persistence	Year of isolation	Pulsotype	Serotype
G62	X	P	2004	C	1/2a
G74	X	P	2004	C	1/2a
G46	XXVII	P	2004	C	1/2a
93	I	P	2005	C	1/2a
98	XIII	P	2005	C	3a
G02	XIV	P	2005	D	1/2a
G05	XIV	P	2005	D	1/2a
GH	X	P	2007	D	1/2a
GI	X	P	2007	D	1/2a
G67	XXI	P	2004	E	1/2a
G39	XI	P	2005	E	1/2a
G65	VIII	NP	2004	A	1/2a
99	III	NP	2005	UNIQUE	3a
GM	XII	NP	2007	UNIQUE	1/2b
GN	XIII	NP	2007	UNIQUE	1/2b
GR	XIV	NP	2007	UNIQUE	1/2a

P: persistent isolate NP: non-persistent isolate.
 Unique pulsotype: pulsotype composed by one isolate.

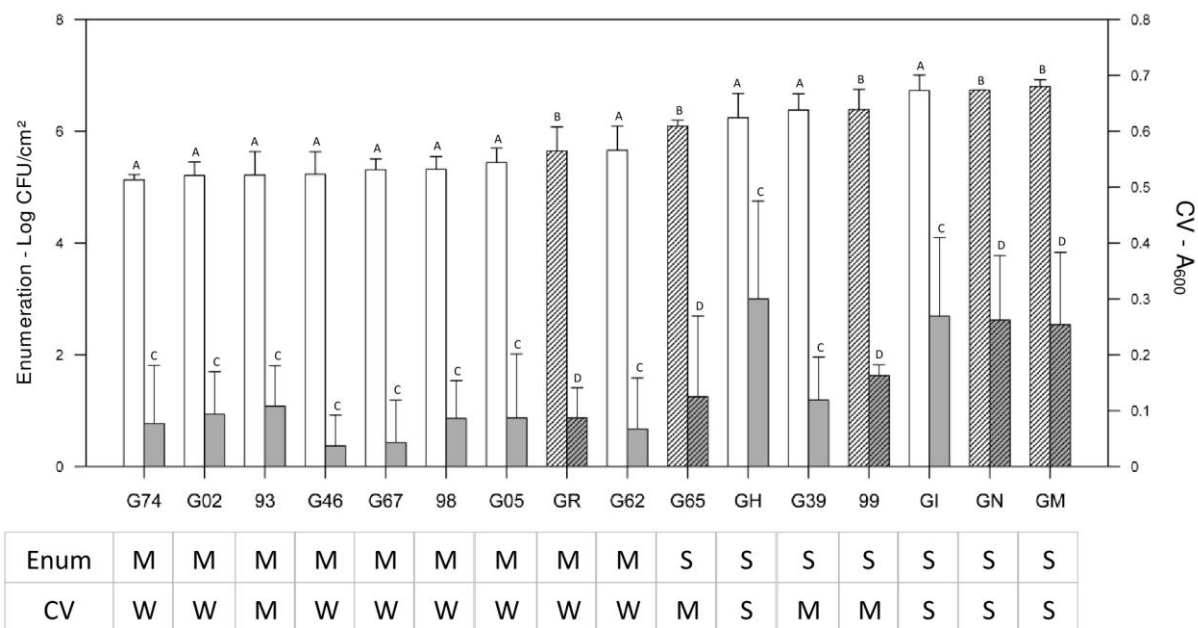


Fig. 1 Biofilm-forming ability assessed by cell enumeration, represented with white bars and the left y-axis, and crystal violet (CV) staining, represented with grey bars and the right y-axis. Biofilms were grown at 25°C for 48 and 24 h, respectively.

Oblique line bars represent non-persistent isolates.

The table under the graph shows the classification in weak, moderate and strong producers:

W- weak producers: 0-0.1 A₆₀₀; 3-4 Log CFU/cm²

M- moderate producers: 0.101-0.2 A₆₀₀; 4.01-5 Log CFU/cm²

S - strong producers: ≥ 0.201 A₆₀₀; ≥ 5.01 Log CFU/cm²

[A-B] Difference between Log CFU/cm² values of persistent (A) and non-persistent (B) isolates: rm-ANOVA p<0.05.

[C-D] Difference between A₆₀₀ values of persistent (C) and non-persistent (D) isolates: rm-ANOVA p>0.05.

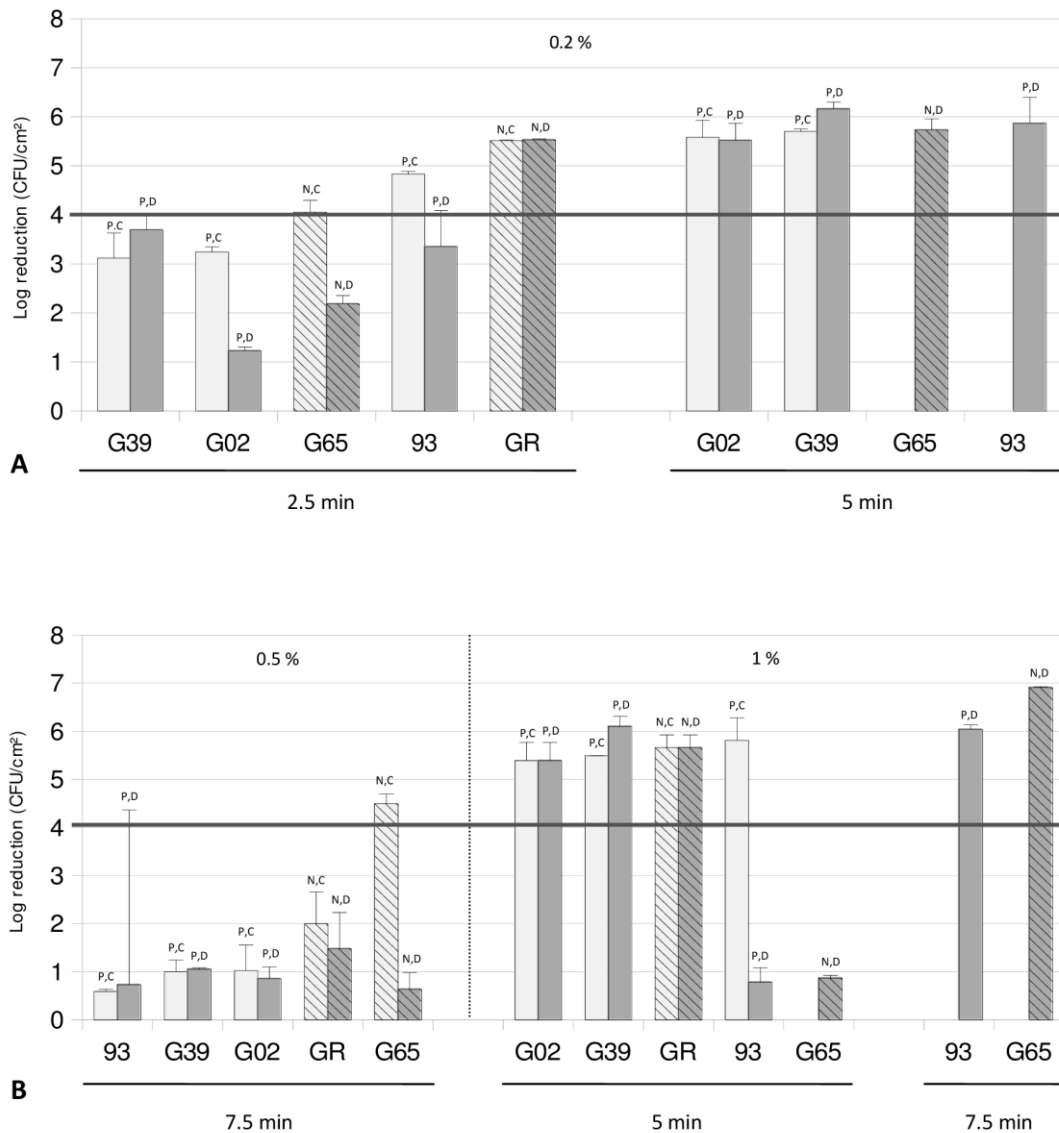


Fig. 2 Disinfectant treatment of *L. monocytogenes* spot inoculated coupons. The Log reduction in viable cell counts are shown after treatment with P3 (A) or MS (B), in clean (light grey bars) and dirty conditions (dark grey bars).

The disinfectant treatment has been considered effective when a 4 Log reduction in viable bacterial counts has been observed. The Log reduction obtained with treatment with MS at 0.5% for 5 min are not shown. Oblique line bars represent non-persistent isolates.

[P-N] Difference between Log reduction of persistent (P) and non-persistent (N) isolates: rm-ANOVA $p > 0.05$.

[C-D] Difference between Log reduction obtained in clean (C) and dirty (D) conditions: rm-ANOVA $p > 0.05$.

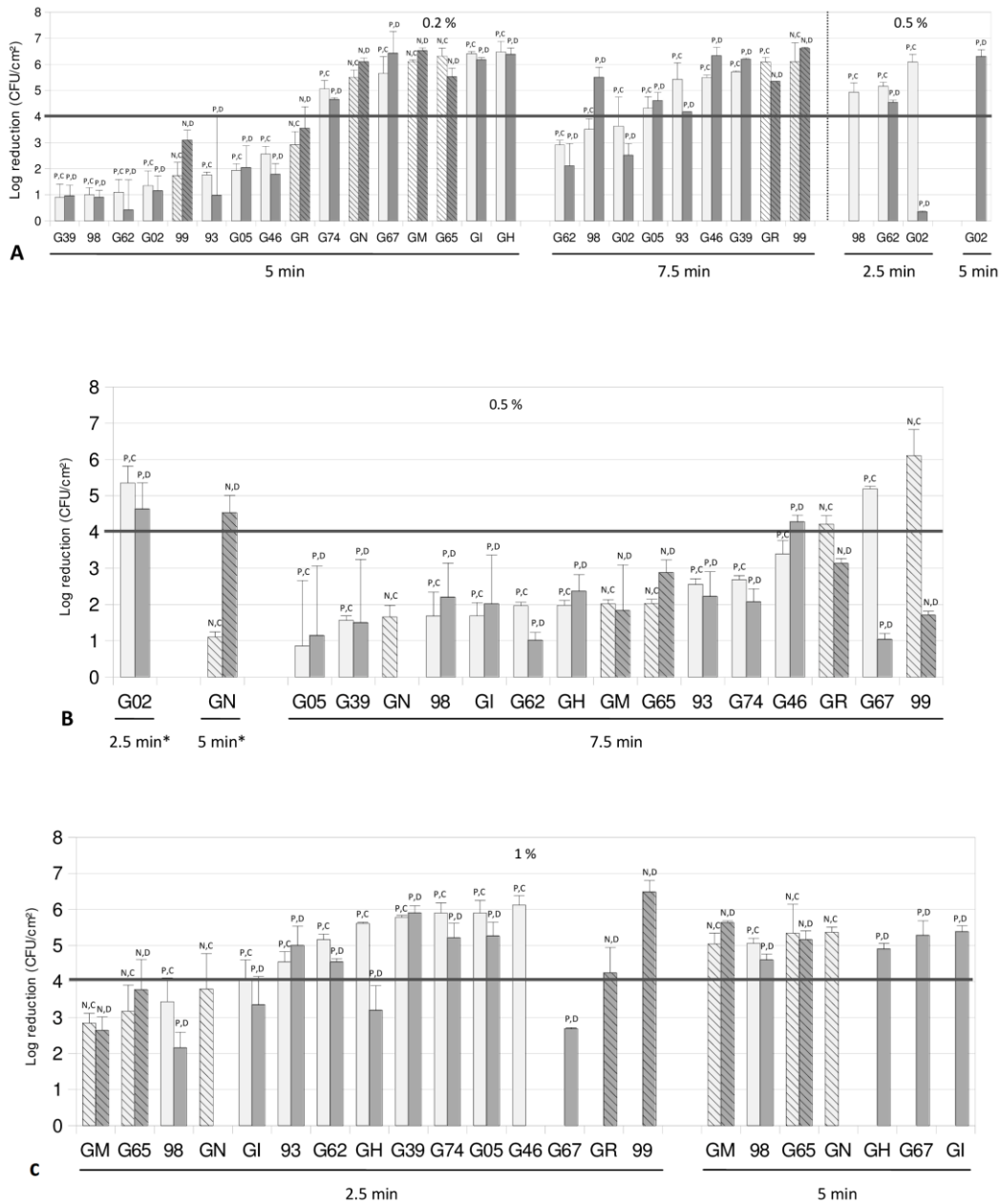


Fig. 3 Disinfectant treatment of *L. monocytogenes* biofilms grown at 25°C for 48 h. The Log reduction in viable cell counts after treatment with P3 in clean (light grey bars) and dirty (dark grey bars) conditions are shown.

The disinfectant treatment has been considered effective when a 4 Log reduction in viable bacterial counts has been observed. The Log reduction obtained with treatment at 0.2% for 2.5 min are not shown. Oblique line bars represent non-persistent isolates.

[P-N] Difference between Log reduction of persistent (P) and non-persistent (N) isolates: rm-ANOVA $p > 0.05$.

[C-D] Difference between Log reduction obtained in clean (C) and dirty (D) conditions: rm-ANOVA $p > 0.05$.

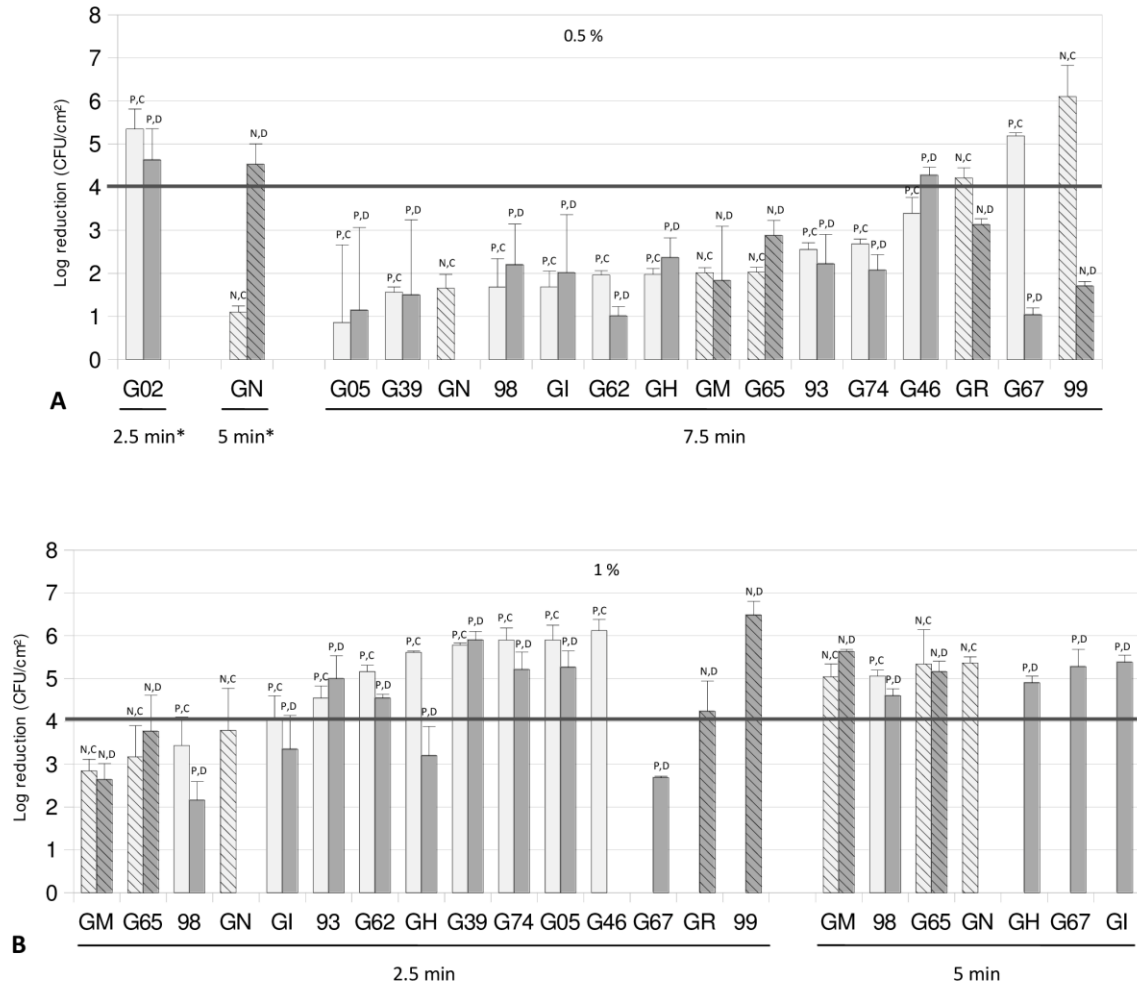


Fig. 4 Disinfectant treatment of *L. monocytogenes* biofilms grown at 25°C for 48 h. The Log reduction in viable cell counts after treatment with MS at 0.5% (A) and 1% (B) in clean (light grey bars) and dirty conditions (dark grey bars) are shown. The disinfectant treatment has been considered effective when a 4 Log reduction in viable bacterial counts has been observed. Oblique line bars represent non-persistent isolates.

* For 2.5 and 5 min contact times the treatments were effective only for the two isolates showed in A.

[P-N] Difference between Log reduction of persistent (P) and non-persistent (N) isolates: rm-ANOVA $p > 0.05$.

[C-D] Difference between Log reduction obtained in clean (C) and dirty (D) conditions: rm-ANOVA $p > 0.05$.