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Behavior of foodborne pathogens, Listeria monocytogenes and Staphylococcus aureus, in mixed-species biofilm exposed to biocides

Oxaran, Virginie; Dittmann, Karen Kiesbye; Lee, Sarah H. I.; Chaul, Luíza Toubas; de Oliveira, Carlos Augusto Fernandes; Corassin, Carlos Humberto; Alves, Virgínia Farias; De Martinis, Elaine Cristina Pereira; Gram, Lone

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1 Behavior of foodborne pathogens, *Listeria monocytogenes* and *Staphylococcus aureus*, in mixed-species
2 biofilm exposed to biocides

3

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13

14

15 **Running title:** Pathogen survival in mixed-species-biofilm

16

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19

20 **Key words**: *Listeria monocytogenes*, *Staphylococcus aureus*, pathogen, mixed-species biofilm, processing
21 environment, biocide

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22 **Abstract**

23 In nature and man-made environments, microorganisms reside in mixed-species biofilm where behavior is
24 modified compared to the single-species biofilms. Pathogenic microorganisms may be protected against
25 adverse treatments in mixed-species biofilms leading to health risk for humans. Here, we developed two
26 mixed-five-species biofilms that included the foodborne pathogens *Listeria monocytogenes* or
27 *Staphylococcus aureus,* respectively. The five species, including the pathogen, were isolated from a single
28 food-processing environmental sample thus mimicking the environmental community. In mature mixed five-
29 species biofilms on stainless steel, the two pathogens remained at a constant level of ~10^5 CFU/cm^2. The
30 mixed-five-species biofilms as well as the pathogens in mono-species biofilms were exposed to biocides to
31 determine any pathogen-protective effect of the mixed biofilm. Both pathogens and their associate microbial
32 communities were reduced by peracetic acid treatments. *S. aureus* decreased 4.6 log cycles in mono-species
33 biofilm, but the pathogen was protected in the five-species biofilm and decreased only 1.1 log cycles. Sessile
34 cells of *L. monocytogenes* were affected equally as a mono-biofilm or as a member in the mixed-species
35 biofilm; decreasing by three log cycles when exposed to 0.0375 % peracetic acid. When the pathogen was
36 exchanged in each associate microbial community, *S. aureus* was eradicated while there was no significant
37 effect of the biocide on *L. monocytogenes* or the mixed community. This indicates that particular members or
38 associations in the community offered the protective effect. Further studies are needed to clarify the
39 mechanisms of biocide protection, and the species playing the protective role in microbial communities of
40 biofilms.

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43 **Importance**

44 This study demonstrates that foodborne pathogens can be established in mixed species biofilms and that this
45 can protect them from biocide action. The protection is not due to specific characteristics of the pathogen,
46 here *S. aureus* and *L. monocytogenes*, but likely caused by specific members or associations in the mixed
47 species biofilm. Biocide treatment and resistance is a challenge for many industries and biocide efficacy
48 should be tested on microorganisms growing in biofilms, preferably mixed systems, mimicking the
49 application environment.

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50 **Introduction**

51 To prevent contamination, infection or foodborne disease in the clinical or food producing sector,
52 antimicrobial, detergents and biocides are used to inactivate or eradicate microorganisms (1, 2). Most
53 guidelines for biocides include testing of efficiency on planktonic pure cultures of microorganisms but little
54 is known about the efficacy on microbial biofilms (3). Moreover, most microorganisms live in complex
55 biofilms (4) composed of multiple species (5, 6). In biofilms, microorganisms can cooperate (7) and protect
56 themselves from adverse environmental conditions. Thus, several studies have reported that sessile cells can
57 be up to 1,000 fold more resistant than cells in a planktonic state (8–10).

58 Biofilm formation and resistance to biocide treatment have been studied and recognized as important
59 factors that contribute to the survival and persistence of microbial contamination in drinking water (11) and
60 in oral hygiene (12, 13). The food processing environment is also believed to provide conditions for biofilm
61 development including polymicrobial biofilms. Although many studies have focused on mono-culture
62 systems, it is being recognized that biofilms are predominantly polymicrobial (14, 15). The behavior of
63 microorganisms in a mixed biofilm differs from the behavior of a mono-species biofilm (16, 17) and for
64 instance resistance to antimicrobials can be increased in the mixed system (18). The complete picture on
65 mechanisms involved in resistance has not been fully unraveled, but some of the reasons for biofilm
66 resistance to antimicrobial compounds are proposed to be caused by the specific architecture, the decreased
67 metabolic activity or the presence of extracellular matrix (19). In addition, conditions in a processing
68 environment such as temperature, have been shown to decrease biocide efficiency on biofilms (20, 21).

69 A major concern raised by the above observations is whether pathogenic microorganisms can be protected
70 in mixed biofilms (22, 23). It has been demonstrated that *Bacillus subtilis*, resistant to peracetic acid
71 exposure, was able to protect *Staphylococcus aureus,* usually sensitive to this disinfectant, in dual-species
72 biofilm (24). Indeed, biofilms in food processing environments have been shown to contribute to foodborne
73 pathogen survival in cleaning and disinfection treatments, leading to persistence of those microorganisms
74 (25).

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75 *Listeria monocytogenes* is a ubiquitous microorganism and it can cause serious foodborne disease. This
76 psychotrophic bacterium has been found in different food products that can lead to listeriosis after ingestion
77 (26). *L. monocytogenes* attaches to surfaces but its ability to form biofilms is controversial (27, 28).
78 Nevertheless, clones of *L. monocytogenes* can survive and persist in niches of processing environments for
79 several years despite cleaning and disinfection procedures (29, 30).

80 *S. aureus* is one of the most common causative agents of food-poisoning and is also involved in
81 nosocomial infections (31). *S. aureus* can form biofilms on different abiotic surfaces found in food
82 processing environment such as glass, stainless steel, polypropylene and polystyrene (32). The food
83 processing environment can also provide suitable conditions for *S. aureus* biofilm production that is
84 enhanced by sub-optimal growth temperature as well as glucose and sodium chloride availability (33).
85 Furthermore, increase of resistance of *S. aureus* in biofilms towards disinfectants in processing environments
86 has been reported in several studies (21).

87 Both pathogens are commonly found in food processing environment including dairy, meat and seafood
88 worldwide (34, 35), and are reported to be common contaminating agents in the Brazilian dairy industry (36–
89 38). Furthermore, several studies have highlighted that the two pathogens are not only found as mono-biofilm
90 but rather in polymicrobial communities (6, 23, 39, 40).

91 The effectiveness of biocides depends on the composition of the food soil, the temperature (3) as well as
92 the antimicrobial used and the surface type (41), the treatment exposure time and the procedure used (10).
93 However, also the biofilm mode of growth is important for effectiveness of biocides. Therefore, the control
94 of biofilm is important for public health in clinical or industrial environments and there is a need for
95 understanding the mechanisms involved in the enhanced pathogen resistance seen in mixed-species biofilm.
96 To date, most studies have focused on mono-species or dual-species biofilms; however, no studies have
97 investigated biocide efficiency using more complex biofilm communities.

98 The purpose of this study was to determine if a “reproducible” mixed-species biofilm model could be
99 established by co-cultivating a pathogen with an associate microbial community isolated from the food

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100 processing sector. Specifically, we sought to determine if foodborne pathogens, such as *S. aureus* and *L.*

101 *monocytogenes*, could establish themselves in such a mixed community and how the presence of this more

102 natural scenario affected their sensitivity to commonly used biocides.

103

104 **Results**

105 **Identification of the community members.** We isolated *L. monocytogenes* and *S. aureus* from two

106 separate samples in Brazilian dairies (42, 43), and subsequently from each of these samples, isolated four

107 different microbial strains at random (Table 1). The sample containing *L. monocytogenes* BZ001 (42) also

108 contained *Klebsiella* sp. (BZ002), *Escherichia coli* (BZ003), *Comamonas* sp. (BZ004) and *Acinetobacter* sp.

109 (BZ006). The sample containing *S. aureus* BZ012 (43) also contained *Aeromonas* spp. (BZ013), *Lactococcus*

110 *lactis* (BZ014), *Candida tropicalis* (BZ017) and *Lactobacillus* sp. (BZ018).

111

112 **Establishment of the pathogen in a dual-species biofilm.** *S. aureus* and *L. monocytogenes* were

113 individually grown in dual-species biofilms with each of their community members (Fig.1). The total sessile

114 cell counts ranged from 3.5 x 10^6 to 3.8 x 10^7 CFU/cm^2 while *L. monocytogenes* counts ranged between 1.8 x

115 10^5 and 1.3 x 10^7 CFU/cm^2 in the dual-species biofilms. The total sessile cell count for the *S. aureus*

116 communities ranged from 3.9 x 10^6 to 9.2 x 10^7 CFU/cm^2 and was between 1.0 x 10^6 and 6.9 x 10^7 CFU/cm^2

117 for the pathogen in the dual-species biofilms.

118

119 **Stable concentration of the pathogen in mixed-five-species biofilm.** In the *S. aureus* mixed-five-species

120 biofilm, the total sessile count was 1.9 x 10^6 CFU/cm^2 and the *S. aureus* sessile cell count was 4.5 x 10^5

121 CFU/cm^2 (Fig. 2). The two different *S. aureus* isolates, BZ012 or Sa30, behaved very similarly in the mixed

122 biofilm (data not shown). The total sessile cell count of the *L. monocytogenes* community was 1.8 x 10^7

123 CFU/cm^2 and 2.3 x 10^5 CFU/cm^2 for *L. monocytogenes* sessile cells. All members of the five species

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124 communities remained in the mature biofilm based on the recovery of all members' colony morphology on
125 BHI plates and by PCR detection for the *S. aureus* community (data not shown).
126
127 **MIC of the biocides.** The MIC of peracetic acid for both pathogens was 0.075 % (1/4 of the concentration
128 used in the dairy) when grown as mono-culture. MIC of peracetic acid against the five-species grown
129 together was 0.075 % for the mixed-culture containing *L. monocytogenes*, and 0.015 % (1/20 of the
130 concentration used in the dairy) for the mixed-culture containing *S. aureus.* The individual MIC of peracetic
131 acid against the associated microorganisms were 0.075 % for the four associate microbial community
132 members of comLm (BZ002, BZ003, BZ004, BZ006), 0.075 % for BZ0013, 0.15% for BZ014 and BZ017
133 and 0.3 % for BZ018.
134 The chlorhexidine digluconate MIC for *L. monocytogenes* was 0.000390625 % and 0.0001953125 % for
135 *S. aureus*. MIC of chlorhexidine digluconate against the five-species of the *L. monocytogenes* associate
136 microbial community grown together was 0.003125 %. The MIC for chlorhexidine digluconate was not
137 determined for the five-species-culture containing *S. aureus* as it was above 0.025 % which was the
138 maximum concentration that could be tested without precipitation.
139
140 **The full community composition influences the biocide susceptibility.** Using the mono- and mixed-
141 five-species biofilm model described above, biocide susceptibility was assessed. The exposure of a mono-
142 biofilm of *S. aureus* to increasing concentrations of peracetic acid led to a sequential decrease of the *S.*
143 *aureus* sessile cell survival (Fig 3A) of 2.5 log between the exposure at 0 % and 0.0375 %, then a 2.1 log
144 reduction between 0.0375 and 0.075 % (MIC value). When *S. aureus* was part of a mixed community
145 biofilms, 0.075 % peracetic acid only caused a 1.1 log reduction of the *S. aureus* sessile cells. This is a
146 significantly ($p = 0.0002$) lower reduction than seen for the mono-biofilm (4.6 log). Increasing the peracetic
147 acid concentration from 0.075 % to 0.15 % fully eradicated *S. aureus* sessile cells from an initial
148 concentration of 4.7 x 10^6 CFU/cm^2 in a mono-biofilm and 5.5 x 10^5 CFU/cm^2 in a mixed-five-species

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149 biofilm (Fig. 3A). At the same time, no associate microbial community member sessile cells were recovered
150 when the mixed-five-species biofilm was exposed to 0.15 % peracetic acid (Fig. 3A), meaning that less than
151 10 CFU/cm^2 were on the SSC if not all eradicated.

152 Treatment of a *L. monocytogenes* mono-biofilm with 0.0375 % peracetic acid caused a 3 log reduction of
153 the sessile cells (Fig. 3B). The same treatment of the mixed-five-species biofilm led to a 2 log reduction of *L.*
154 *monocytogenes* sessile cells while the total sessile cells decreased by 3.7 log. When exposed to higher
155 concentration of peracetic acid *e. g.* 0.075 %, no viable *L. monocytogenes* sessile cells were recovered from a
156 mono-biofilm. The total sessile cells and the *L. monocytogenes* sessile cells decreased by 0.8 log and 0.3 log,
157 respectively, in the mixed-five-species biofilm treated with 0.075 % peracetic acid compared to treatment
158 with 0.0375 % (Fig. 3B). No viable sessile cells were recovered when *L. monocytogenes* was grown as a
159 mono-species biofilm or in a mixed-five-species biofilm treated with 0.15 % peracetic acid (2 fold higher
160 than the MIC) (Fig. 3B).

161

162 **No effect of chlorhexidine digluconate was observed.** In order to assess susceptibility to other biocides
163 used in the processing environment, the effect of chlorhexidine digluconate was also evaluated (Fig. 4). No
164 effect of chlorhexidine treatment was observed on *S. aureus* grown as mono-biofilm or as part of mixed-five-
165 species biofilm (Fig. 4A).

166 There was no impact of chlorhexidine digluconate treatment on *L. monocytogenes* for concentrations
167 below 0.0125 % (Fig. 4B) whenever the pathogen was grown as a mono-biofilm or as part of a mixed-five-
168 species biofilm. *L. monocytogenes* sessile cell counts remained steady in a mono-biofilm with 10^4 CFU/cm^2
169 when treated with concentration below 0.0125 % of chlorhexidine digluconate while it decreased from 1.6 x
170 10^3 CFU/cm^2 to 1.2 x 10^2 CFU/cm^2 in a mixed-five-species biofilm (Fig. 4B). This 1.2 log reduction was
171 observed in the mono-biofilm when the sessile cells were exposed from 0.0125 % to 0.25 % chlorhexidine
172 digluconate. The concentration of the total sessile cells, being of 4 x 10^6 CFU/cm^2, did not change when

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173 exposed to 0.00625 % and 0.0125 %, but decreased 1.3 log when exposed to 0.025 %. Concentrations higher
174 than 0.025 % were not investigated due to technical limitations, as the chlorhexidine digluconate precipitated.
175
176 **The community rather the individual influences peracetic acid susceptibility.** To determine if the
177 altered sensitivity to peracetic acid in a mixed species biofilm was due to the associate microbial community
178 or to the pathogen, we interchanged the pathogens and the communities. When *S. aureus* was grown with the
179 *L. monocytogenes* community members, on the SSC not treated with peracetic acid, the total sessile cells
180 count was 1.3 x 10^8 CFU/cm^2 and *S. aureus* sessile cells count was 2.5 x 10^4 CFU/cm^2 (Fig. 5A). No cells
181 were recovered after treatment with 0.075% peracetic acid indicating that the total sessile cells decreased of
182 at least 6.3 log ($p = 0.001$) and the pathogen sessile cells decreased of at least 3.1 log ($p < 0.001$). When *L.*
183 *monocytogenes* was grown with the *S. aureus* community members, the overall biofilm production on SSC
184 did not change (Fig. 3B and 5B). Treatment with 0.075 % peracetic acid led to a reduction of the total sessile
185 cells by 3.4 log ($p < 0.001$) and to 1.9 log of the *L. monocytogenes* sessile cells (Fig. 5B).
186

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187 **Discussion**

188 In environments, either natural, clinical or industrial, microorganisms mainly live in biofilms that are well
189 structured communities and mostly composed of more than one microbial species (5, 6). However, in natural
190 or man-made environment, the quantity and diversity of present species raise the complexity underlying the
191 behavior of the biofilms compared to their single species growth behavior (7). Pathogenic microorganisms
192 can also form biofilm or inhabit biofilms and this can cause problems for human health, especially if biocide
193 treatment is not fully effective. Also, survival of microorganisms after biocide exposure is one of the results
194 of the behavior modification. To date, most biofilm studies focusing on their mechanistic properties or
195 antimicrobial resistance have been done on mono-species biofilms which do not reflect the complexity
196 reached in mixed-community. Recently, some studies on mixed-biofilms have also been conducted but they
197 have been limited on species diversity (44–47) without focusing on a specific pathogenic organisms.

198 In this study, we set up and studied two mixed-five-species biofilms containing a pathogenic bacterium.
199 The ability of some strains to form biofilm fluctuated with some dual-species showing better ability to form
200 biofilm than others depending on the strain association. Norwood *et al*. (48) have shown that *Pseudomonas*
201 *fragi* and *S. xylosus* were the predominant species in biofilm with *L. monocytogenes*, and they described, as
202 have other studies, that some microorganisms can take over in mixed-species biofilms. However, no strains
203 were inhibited in our study. Both in the dual-species and the mixed-five-species biofilms, all strains remained
204 in the mature biofilm, including both pathogens. A previous study on mixed-biofilm composed of *L.*
205 *monocytogenes* and *Enterococcus* spp. found that growth at 25°C on stainless steel gave the highest biofilm
206 cell counts (44), supporting the temperature selection that was used in this present study. Furthermore, the
207 mixed-five-species biofilm model here was stable and reproducible; leading to a biofilm tool which can be
208 used to study the behavior of pathogen in mixed-biofilms and to unravel mechanisms involved in survival or
209 persistence towards antimicrobial compounds such as biocides.

210 *L. monocytogenes* is commonly described as non-/weak mono-layered biofilm producer (23, 49) but can
211 colonize and persist in mixed-species biofilms (23) as also found in the present study. Some authors have

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212 described that *L. monocytogenes* was inhibited in biofilms composed of several bacteria as compared to
213 mono-biofilm (40, 48, 50), *e. g. S. sciuri* prevented *L. monocytogenes* from adhering and being part of a
214 biofilm (50). In contrast, the sessile cell counts of *L. monocytogenes* on stainless steel were similar in mono-
215 and mixed-five-species biofilms in this study, which is in accordance with other studies (40, 46, 51).

216 *S. aureus*, the other major pathogen selected is a renowned biofilm former (52), also in multispecies
217 biofilms with *P. aeruginosa* (53, 54), *B. subtilis* (55), *E. faecalis* (56) or *C.albicans* (53, 57). Some studies
218 have reported that lactic acid bacteria such as *Lactococcus* spp. and *Lactobacillus* spp. (58, 59) can inhibit
219 growth of this pathogen while that was not noticed in this study. However, the sessile cell counts of each of
220 the associate microbial community member including the pathogen decreased in the mixed-five-species
221 biofilms compared to the sessile cell counts in mono- or dual-species biofilms which could be due to
222 competition for nutriment and antimicrobial compound production (59).

223 Here, dairy sample communities were composed of various microorganisms representing the diversity
224 existing in natural and man-made environment. Several studies have demonstrated that processing
225 environments are composed of a large diversity of microorganisms (60, 61) and Dzieciol *et al.* (60) have
226 shown that microbial communities were distinct depending on the collecting point, as observed in this study.
227 Hence, it could be suggested that the microbial diversity in processing environments leading to the biofilm
228 diversity, could impair effective disinfection procedure as Simões *et al.* (47) also concluded.

229 To date, very few studies have investigated antimicrobial resistance of mixed-species biofilms (46, 48)
230 including comparison with mono-species biofilm data. Using the mature mixed-five-species biofilms on
231 stainless steel coupon set up in this study, the biofilm models were subjected to biocides similar to those used
232 in the processing environment where the strains were sampled. The effectiveness of biocide treatment on the
233 two pathogens depended on the biocide used as previously shown (8, 44, 62). Peracetic acid treatment
234 impaired survival in mono- and mixed-five-species biofilms while no significant effect of chlorhexidine
235 digluconate was seen at the tested concentrations. Da Silva Fernandes *et al.* (44) have described peracetic
236 acid as the most efficient biocide used in processing environment compared to quaternary ammonium,

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237 sodium hypochlorite or biguanide and it was able to eliminate *L. monocytogenes* from the multi-species
238 biofilm as observed in this study at a concentration of 0.15 %. Our results indicate that peracetic acid as used
239 in the dairies (0.3 %) is efficient in reducing bacterial numbers since less than 10 CFU/cm^2 of sessile cells
240 were recovered after treatment with 0.15 % peracetic acid (1/2 of the factory in-use concentration). The effect
241 of chlorhexidine digluconate cannot be evaluated since the biocide precipitated at concentrations above 0.025
242 %.

243 Noteworthy, sessile cells of *S. aureus* were recovered from both mono- and mixed-five-species biofilm
244 (comSa), when treated with 0.075 % peracetic acid. Also, *S. aureus* sessile cells survived in a mono-species
245 biofilm at this concentration which was the MIC determined for this pathogen. This corroborates that
246 biofilms can modify and increase biocide susceptibility response as suggested by some authors (8).

247 The mixed associate microbial communities, comSa or comLm, protected the pathogens during peracetic
248 acid treatment. Thus, a 1 log difference was obtained between sessile cell counts of *L. monocytogenes* in
249 mono- and mixed-five-species biofilms after biocide treatment. Using the same biocide, Van der Veen and
250 Abee (63) also observed that *L. monocytogenes* was more resistant to peracetic acid in mixed-biofilm with *L.*
251 *plantarum* than in mono-species biofilm. In the case of *S. aureus* sessile cells, a decreased of 2.1 log was
252 obtained between treatment at 0.0375 % and 0.075 % peracetic acid of the mono-species biofilm while in a
253 mixed-five-species biofilm the decrease was only of 0.2 log. Hence, similarly to Bridier *et al.*(55), *S. aureus*
254 was less susceptible to peracetic acid exposure when the pathogen was part of a mixed-biofilm compared to
255 growth as a mono-species biofilm.

256 We investigated if the increase of pathogen survival in mixed-species biofilm was due to specific ability
257 of this pathogen in the associate microbial community or to the community itself. We interchanged the
258 pathogens and the associate microbial communities *i.e. S. aureus* with comLm and *L. monocytogenes* with
259 comSa. The new mixed-five-species biofilms were only treated with 0.075 % peracetic acid which was the
260 highest concentration that allowed survival sessile cells recovery in the first set of experiments. *S. aureus* was
261 established at a lower density in comLm than in the original comSa while the total sessile cell counts

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262 increased. However, *S. aureus* was not protected by the comLm community and no *S. aureus* sessile cells
263 were recovered. In the biofilm composed of *L. monocytogenes* with comSa and treated with 0.075% peracetic
264 acid, a slight increase of survival sessile cells of *L. monocytogenes* was recovered compared to the
265 association with comLm. This could be due to microbial composition variation, comLm being only
266 composed of Gram negative bacteria while comSa was composed of Gram positive and negative bacteria as
267 well as one yeast. Therefore, it is likely the full associate microbial community which is involved in the
268 survival/resistance mechanisms. This protective effect could be due to the microbial species composition
269 which influences the biofilm matrix composition as well as the presence of specific microorganisms with
270 higher resistance to the tested biocide *e.g.* the MIC for peracetic acid of each member of comLm was 0.075%
271 whereas members of comSa showed different MIC value ranging from 0.075 % to 0.3 % (concentration used
272 in dairy). This is in agreement with the suggestion (64) that biocide resistance could be due to the
273 extracellular polymeric substance of the matrix or to the environment but not only on the species specific
274 attributes (46).
275
276 In conclusion, biofilms can vary due to the microbial diversity encountered in man-made environments.
277 This diversity is a key challenge to eradicate unwanted microorganisms. Further studies are required to
278 unravel the exact mechanisms leading to the protective role of the community using complex microbial
279 biofilm mimicking biofilms encountered in industry/clinical or natural environment as we attempted to set up
280 in this study. Therefore, by improving the knowledge on mixed-species biofilm behavior in the presence of
281 biocides, control of biofilm could be improving in any kind of sector.

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282 **Materials and Methods**

283 **Bacterial strains and growth media and biocides.** The strains of *L. monocytogenes* and *S. aureus* used
284 in this study have been isolated from Brazilian dairies (42, 43) (Table 1). Four of the associate microbial
285 community were also isolated as described below. They were isolated from the same sample as the
286 pathogenic bacterium to obtain two communities as encountered in the dairy: one containing *L.*
287 *monocytogenes* and one containing *S. aureus* (Table 1).

288 Two isolates of *S. aureus*, BZ012 and Sa30, were used in this study. The isolate BZ012 was selected
289 representing the sequence type ST398 (43), a major possible health risk ST, and was used for dual-biofilm
290 and mixed-species biofilm setup experiments. The isolate Sa30 was chosen as it represented the major ST/CC
291 trend, ST30/CC1, found in the Brazilian dairy industry (43), and this isolate was used for assessing biocide
292 effect on biofilm containing *S. aureus* (including the MIC assay). It was rationalized that the associate
293 microbial community members isolated from the sample containing *S. aureus* BZ012 would be suitable for
294 any *S. aureus* dairy isolates and therefore they were also used as community members with the *S. aureus*
295 Sa30 isolate in the biocide susceptibility experiments.

296 Isolates were grown on Brain-Heart Infusion (BHI) broth (Oxoid), BHI agar (BHI broth, 1.5% agar,
297 AppliChem) or Tryptic Soy Agar (TSA, Oxoid), Man Rogosa and Sharpe agar (MRS) and Dichloran Rose
298 Bengal Chloramphenicol agar (DRBC, Oxoid, UK) and Potato Dextrose Agar plus chloramphenicol (PDA-
299 CAM, Oxoid, UK). *S. aureus* was enumerated on Baird Parker agar (Oxoid, UK) with egg yolk emulsion
300 (Oxoid, UK) and *L. monocytogenes* was counted on Oxford (Oxoid, UK) with modified *Listeria* selective
301 supplement (Oxoid, UK). Unless otherwise specified, isolates were grown at 37°C and liquid cultures were
302 incubated under shaking conditions at 250 rpm. The isolates were stored in BHI containing 20 % (vol/vol)
303 glycerol (Merck, Germany) at -80°C. For biocide treatments, two biocides used in the dairies from where
304 samples were taken were used: peracetic acid (Sigma-Aldrich, Germany) and chlorhexidine digluconate
305 (Sigma-Aldrich, Germany). These stock solutions were diluted in NaCl 0.9 % (w/v) to the selected
306 concentrations.

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307 **Selection and identification of four associated microorganisms for each pathogen**

308 ***Selection of 4 associated microorganisms for each pathogen by phenotypical tests.*** Using the same
309 samples where the *L. monocytogenes* BZ001 (42) and *S. aureus* BZ012 (43) were isolated, four associate
310 community members were isolated by surface platting 0.1 mL of a tenfold diluted sample in peptone water
311 0.1% supplemented with NaCl 0.85% (Oxoid, UK) suspension on BHI agar, MRS and DRBC. MRS and BHI
312 agar plates were incubated at 30°C for 24 to 48 hours. DRBC plates were incubated at 25°C up to 7 days and
313 then purified on PDA-CAM. Up to three different colonies of different morphologies were selected and
314 purified on the respective culture media for subsequent analysis. Only one community *e.g.* four associate
315 community members, for each pathogen species was selected and used in this study.

316 ***Species identification.*** The isolates were identified by phenotypic characterization and 16S rRNA or 28S
317 rDNA gene sequencing. Shape and motility were determined by microscopy using an Olympus microscope
318 (BX51). Gram-reaction was assessed by the 3% KOH method (65). Catalase or cytochrome oxidase was
319 tested using 3 % hydrogen peroxide (Merck, Germany) and dry slide (BD Diagnostics, NJ, USA),
320 respectively. DNA manipulation and 16S rRNA gene sequencing using the primer couple 27F (66) and
321 1492R (67) or NL1 (68) and LS2 (69) for the 28S rDNA were performed as described in Oxaran *et al.* (42)
322 (Table 2).

323 **Development of mature biofilms on stainless steel coupon.** In order to reproduce the dairy processing
324 environment, biofilm formation was assessed on stainless steel coupons (SSC) at 25°C (temperature noticed
325 in the investigated dairies) using an associate microbial community of strains isolated from Brazilian dairies
326 (42, 43). Overnight cultures were inoculated to a final OD_{600nm} of 0.01 in a 5 mL BHI broth tube containing a
327 1 x 2 cm SSC (AISI 316, unpolished, 2B finish, prepared according to Kastbjerg and Gram (70)). Mono-
328 species biofilms were produced from only one single strain, dual-species biofilms were composed of the
329 pathogenic strain (*L. monocytogenes* or *S. aureus*) and one of the associate microbial community members,
330 and mixed-five-species biofilms were obtained by the association of a pathogen strain and the four associate
331 microbial community members listed in Table 1 (each inoculated at an equal ratio). Adhesion was performed

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332 for 1 h 30 min at 25°C, with shaking at 90 rpm. Subsequently, the media was gently discarded to remove
333 planktonic and loosely attached cells and replaced with BHI broth followed by incubation at 25°C, with
334 shaking at90 rpm. To obtain a mature biofilm, the *S. aureus*-containing biofilm was incubated for 25 h and
335 the *L. monocytogenes*-containing biofilm for 72 h.

336 **Enumeration of planktonic and sessile cells.** Viable sessile cells in the mature biofilm on SSC were
337 enumerated by determination of colony forming units (CFU). The SSC was gently washed three times with 2
338 mL NaCl 0.9 % and subsequently sonicated in 2 mL NaCl 0.9 % for 2 min (DELTA 220T, Aerosec
339 Industrie). The CFU count of viable sessile cells was assessed by the drop plating method on BHI agar for the
340 total cell count (including all the associate microbial community members), and for the model pathogen cell
341 count, selective media was used *i. e.* BP or Oxford for *S. aureus* and *L. monocytogenes*, respectively. As
342 growth control, the planktonic cell counts were done using the same method. The results considered the
343 detection limit of the method used in this study which was 10 CFU/cm^2.

344 **Species presence assessment.** To check that all associate microbial community members remained in the
345 mature biofilm, two methods were used. For the community containing *L. monocytogenes*, species presence
346 was evaluated by colony morphology on BHI agar, as the five community members showed distinct colony
347 morphology. For the community containing *S. aureus*, colonies were not distinguishable on agar plate and
348 PCR reaction using species specific primers (Table 2) was used to detect the different organisms. Genomic
349 DNA (gDNA) was extracted using the NucleoSpin® Tissue Kit (Macherey-Nagel, Germany) from the sessile
350 cells using the same suspension used for enumeration, following the manufacturer´s recommendations. PCR
351 reactions were performed using the TEMPase Hot Start 2x Master Mix Blue II (Ampliqon, Denmark)
352 according to the manufacturer's protocol. One µL of gDNA was added as matrix. The PCR reaction was run
353 in a Veriti Thermal Cycler (Applied Biosystems, 96 Well Model 9902) and amplicon presence was checked
354 by gel electrophoresis.

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355 **MIC of biocides.** A first assessment of biocide susceptibility was performed using Minimum Inhibitory
356 Concentration (MIC) assays in order to target concentrations to be used in the biofilm model. The
357 concentrations used in the dairies were 0.3 % and 0.04 % for peracetic acid and chlorhexidine digluconate,
358 respectively (S. H. I. Lee, personal communication). Each pathogen (*L. monocytogenes* and *S. aureus* Sa30)
359 was assessed by MIC assay as mono-culture and mixed-culture (with the four associated microorganisms).
360 The MIC assay was also done for the individual community members following the same protocol. Overnight
361 cultures were adjusted to OD_{600nm} = 0.02 in BHI and 100 µl were dispensed into wells (96-well, round-
362 bottom microtiter plate, Thermo Scientific, MA, USA) containing 100 µl of a serial dilution of each biocide.
363 Final biocide concentrations tested started at 4.8 % and decremented by 2 up to 13 times for peracetic acid
364 and at 0.05 % and decremented by 2 up to 9 times for chlorhexidine digluconate. The MIC was determined
365 after incubation for 24 h at 25°C, shaking at 90 rpm and was determined as the minimum concentration
366 inhibiting growth.

367 **Biocide treatment of mature biofilm on stainless steel coupons.** After development of mature mono- or
368 mixed-five-species biofilms, sessile cells were subjected to disinfection treatment. SSC were washed two
369 times in 2 mL NaCl 0.9 % containing plates (6-well plate, Nunc, Denmark) and then immersed in a 2 mL
370 NaCl 0.9 % solution at the selected biocide concentrations. Biocide treatment was stopped after 20 min of
371 exposure (70) at 25°C, shaking at 90 rpm by transferring the SSC to 2 mL of Dey-Engley neutralizing broth.
372 Then, the SSC was sonicated for 2 min in the neutralizing solution and enumeration was performed as
373 described above.

374 **Statistic data analysis.** Statistical analyses were performed using the Excel Data Tool to process all data
375 by variance analysis (ANOVA). Significance was defined with a Fisher test value as a P value ≤ 0.05.

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386

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571 **Figure legends**

572 **FIGURE 1**

573 Quantification of viable sessile cells on dual-species biofilms. Each pathogen, (A) *L. monocytogenes*
574 (BZ001) or (B) *S. aureus* (BZ012) were grown as mono- or dual-biofilm developed on SSC with each of the
575 associated community members. At least three biological replicates for *L. monocytogenes* and only duplicates
576 for *S. aureus* were done.

577

578 **FIGURE 2**

579 Sessile cell counts of mono- and mixed-five-species mature biofilms. Pathogens were *L. monocytogenes*
580 (BZ001) or *S. aureus* (Sa30). The mature biofilm was a 25 h biofilm and a 72 h biofilm for the *S. aureus*
581 community and the *L. monocytogenes* community, respectively. Pathogen cell count was assessed on Baird
582 Parker agar for *S. aureus* and Oxford agar for *L. monocytogenes.* Total community cell count was assessed on
583 BHI agar. Error bars represent the standard deviation of three biological replicates.

584

585 **FIGURE 3**

586 Survival of sessile cells in biofilm formed on SSC after peracetic acid exposure. For each pathogen, (A) *S.*
587 *aureus* (Sa30) or (B) *L. monocytogenes* (BZ001), mono- and mixed-five-species biofilms were developed on
588 SSC as mature biofilm before being treated with different concentrations of peracetic acid (PA) for 20 min.
589 Sa30: mono-biofilm of *S. aureus*; Sa30-comSa: mixed-five-species biofilms containing Sa30; BZ001: mono-
590 biofilm of *L. monocytogenes*; BZ001-comLm: mixed-five-species biofilms containing BZ001. Results are
591 presented as the mean of biological triplicates.

592

593 **FIGURE 4**

594 Survival of sessile cells in biofilm formed on SSC after treatment with different concentrations of

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595 chlorhexidine digluconate for 20 min. For each pathogen, (A) *S. aureus* (Sa30) or (B) *L. monocytogenes*
596 (BZ001), mono- and mixed-five-species biofilms were developed on SSC as mature biofilm before treatment.
597 Sa30: mono-biofilm of *S. aureus*; Sa30-comSa: mixed-five-species biofilms containing Sa30; BZ001: mono-
598 biofilm of *L. monocytogenes*; BZ001-comLm: mixed-five-species biofilms containing BZ001. Results are
599 presented as the mean of biological triplicates for *L. monocytogenes* (B) and only one biological replicate for
600 *S. aureus* (A).
601
602 **FIGURE 5**
603 Survival of sessile cells in mixed-species biofilm after 20 min with 0.075% peracetic acid (PA) treatment.
604 Each pathogen, (A) *S. aureus* (Sa30) or (B) *L. monocytogenes* (BZ001) were grown as mixed-five-species
605 biofilm developed on SSC with the other pathogen community members, *i.e.* BZ001 associated with comSa
606 and Sa30 associated with comLm. Sa30-comLm: mixed-five-species biofilm containing Sa30; BZ001-com:
607 mixed-five-species biofilm containing BZ001. Results are presented as the mean of biological triplicates.
608

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1 **TABLE 1** Bacterial strains used in the present study.

Pathogen / Community	Background microorganisms	Name	Reference
L. monocytogenes		BZ001	(52)
comLm	*Klebsiella* spp.	BZ002	This study
	E. coli	BZ003	This study
	Comamonas spp.	BZ004	This study
	Acinetobacter spp.	BZ006	This study
S. aureus		BZ012	(53)
		Sa30	(53)
comSa	*Aeromonas* sp.	BZ013	This study
	Lactococcus lactis	BZ014	This study
	Candida tropicalis	BZ017	This study
	Lactobacillus sp.	BZ018	This study

2

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4 **TABLE 2** Primers for species identification.

Species	Primer	Sequence (5'-3')	Reference
	27F	AGAGTTTGATCCTGGCTCAG	(87)
	1492R	GGTTACCTTGTTACGACTT	(88)
	NL1	GCCATATCAATAAGCGGAGGAAAAG	(89)
	LS2	ATTCCCAAACAACTCGACTC	(90)
S. aureus	16S-Saureus-F	ATACAAAGGGCAGCGAAACC	This study
	16S-Saureus-R	TCATTTGTCCCACCTTCGAC	This study
L. lactis	16S-Llactis-F	AAGTTGAGCGCTGAAGGTTG	This study
	16S-Llactis-R	AACGCGGGATCATCTTTG	This study
Lactobacillus spp.	16S-Lbacillus-F	ATTTTGGTCGCCAACGAG	This study
	16S-Lbacillus-R	ACCCCACCAACAAGCTAATG	This study
Aeromonas spp.	16S-Aerom-F	GCAGCGGGAAAGTAGCTTG	This study
	16S-Aerom-R	ATATCCAATCGCGCAAGG	This study
C. tropicalis	16S-Ctropicalis-F	GTCGAGTTGTTTGGGAATGC	This study
	16S-Ctropicalis-R	ATCTCAAGCCCTTCCCTTTC	This study

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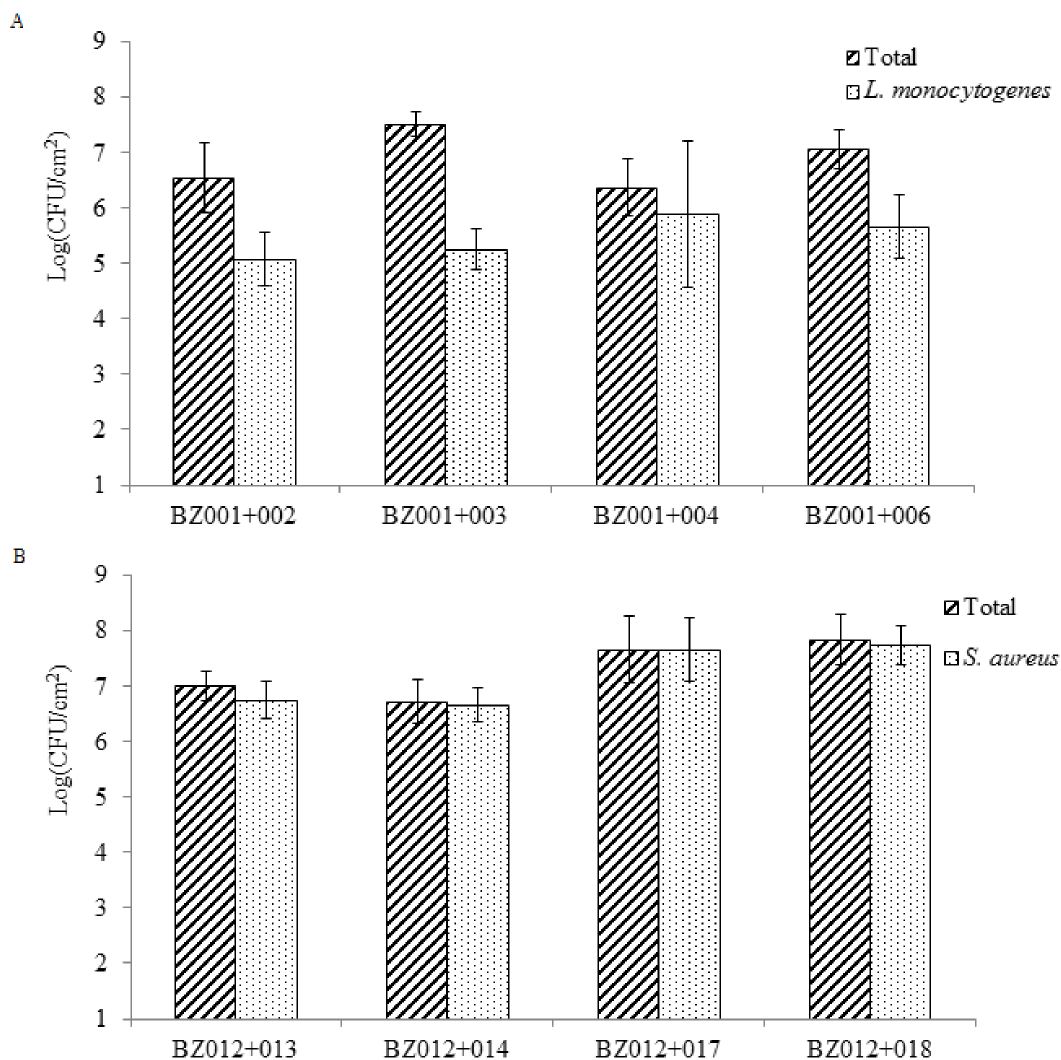

1

2 **FIGURE 1**

3 Quantification of viable sessile cells on dual-species biofilms. Each pathogen, (A) *L. monocytogenes*
4 (BZ001) or (B) *S. aureus* (BZ012) were grown as mono- or dual-biofilm developed on SSC with each of the
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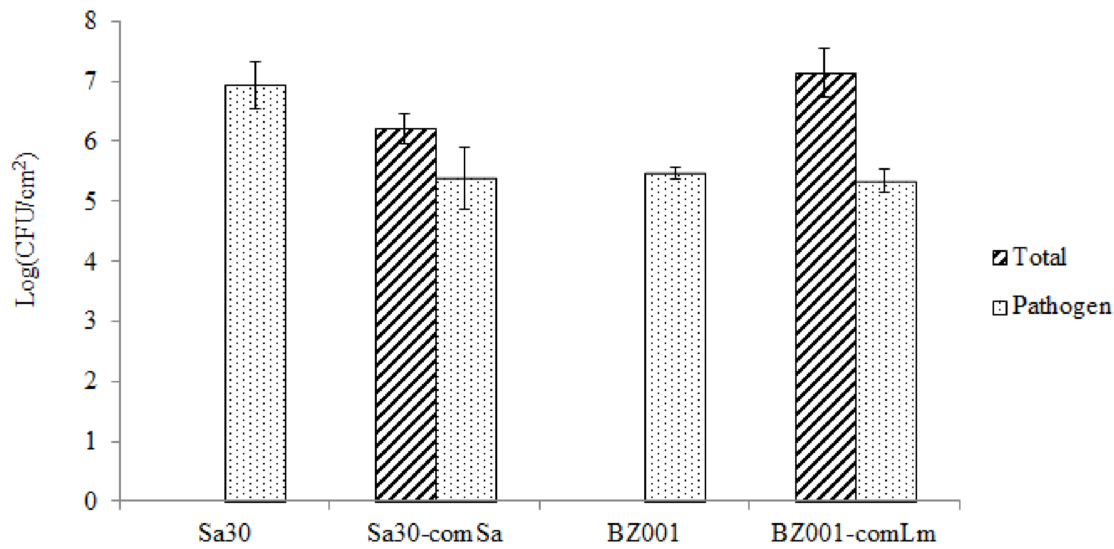

8

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10 Sessile cell counts of mono- and mixed-five-species mature biofilms. Pathogens were *L. monocytogenes*
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13 Parker agar for *S. aureus* and Oxford agar for *L. monocytogenes.* Total community cell count was assessed
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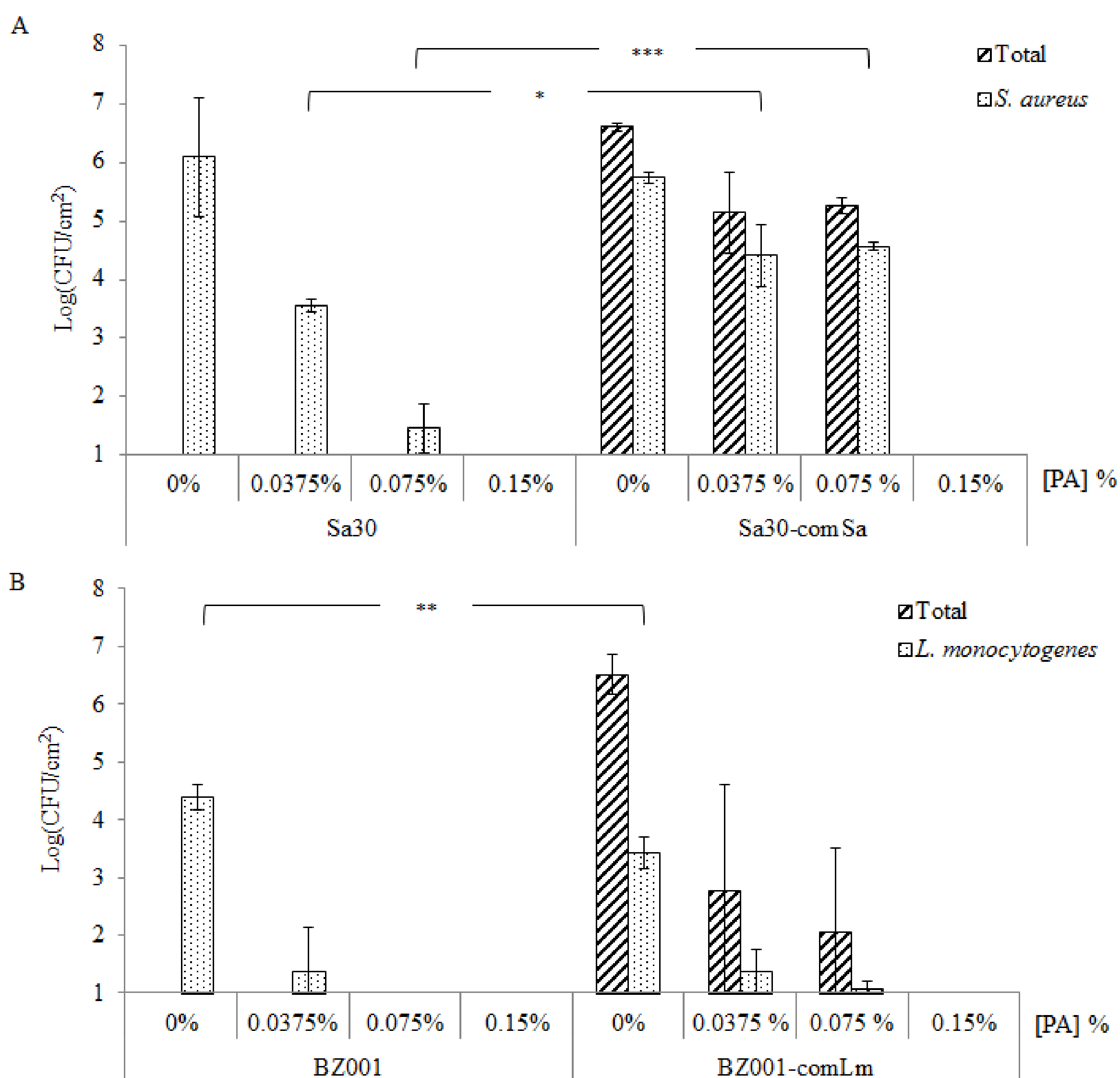

15

16 **FIGURE 3**

17 Survival of sessile cells in biofilm formed on SSC after peracetic acid exposure. For each pathogen, (A) *S.*
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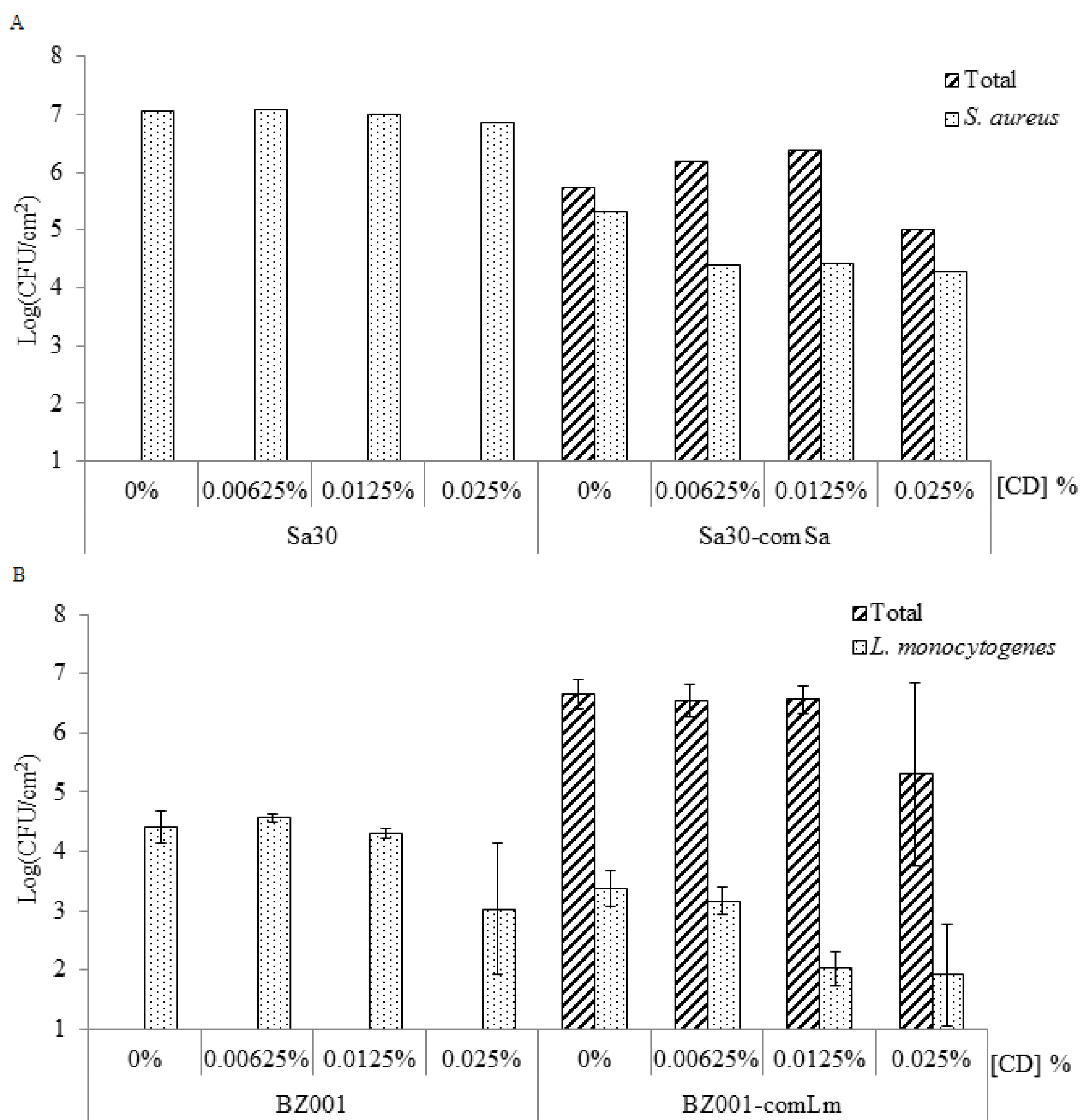

23

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25 Survival of sessile cells in biofilm formed on SSC after treatment with different concentrations of

26 chlorhexidine digluconate for 20 min. For each pathogen, (A) *S. aureus* (Sa30) or (B) *L. monocytogenes*

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29 biofilm of *L. monocytogenes*; BZ001-comLm: mixed-five-species biofilms containing BZ001. Results are

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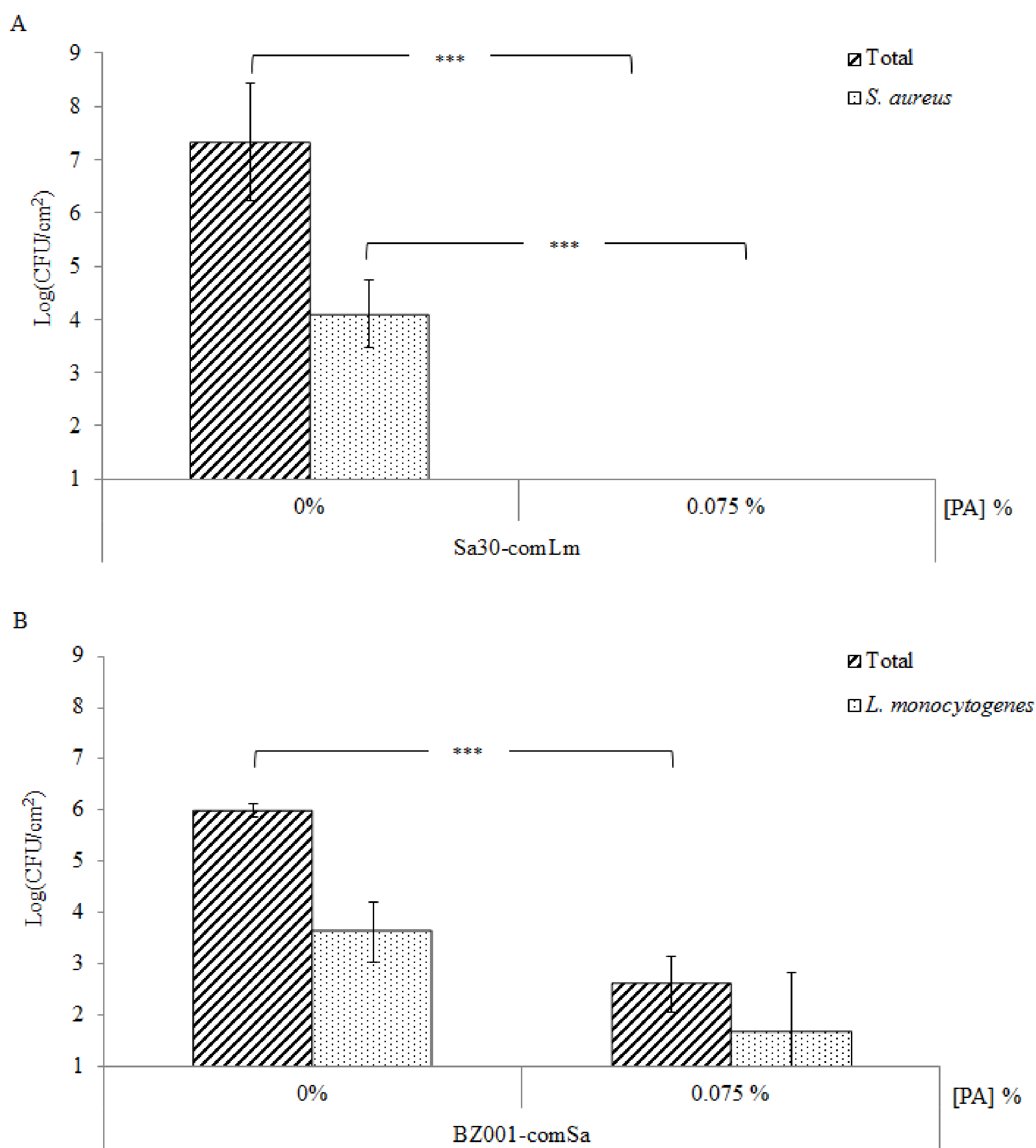

31

32 **FIGURE 5**

33 Survival of sessile cells in mixed-species biofilm after 20 min with 0.075% peracetic acid (PA) treatment.

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