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Research Letter

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Control of *Listeria monocytogenes* biofilms on industrial surfaces by the bacteriocin-producing *Lactobacillus sakei* CRL1862

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One sentence summary: This work described the ability of the bacteriocinogenic *Lactobacillus sakei* CRL1862 to control *Listeria monocytogenes* biofilm on industrial surfaces at 10°C.

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ABSTRACT

The effect of the bacteriocin-producing *Lactobacillus sakei* CRL1862 and its bacteriocin in the control of *Listeria* biofilm formation on industrial surfaces at 10°C was investigated. A screening among different *Listeria* species was performed allowing selecting *L. monocytogenes* FBUNT for its use as a biofilm producer on stainless steel (SS) and polytetrafluoroethylene (PTFE) surfaces. Three conditions were simulated to evaluate the ability of the bacteriocinogenic strain to displace, exclude and compete pathogen biofilm formation. *Lactobacillus sakei* CRL1862 effectively inhibited biofilm formation by *L. monocytogenes* FBUNT through the three assayed mechanisms, pathogen inhibition being more efficient on PTFE than on SS surface. Moreover, co-culture of *L. monocytogenes* FBUNT with the bacteriocin-producer displayed the highest efficacy reducing the pathogen by 5.54 ± 0.12 and 4.52 ± 0.01 on PTFE and SS, respectively. Industrially, the pre-treatment with *L. sakei* CRL1862 or its bacteriocin (exclusion) constitutes the most realistic way to prevent pathogen biofilm settlement. The use of bacteriocins and/or the bacteriocin-producer strain represents a safe and environmentally-friendly sanitation method to mitigate post-processing food contamination.

Keywords: biofilm; *Listeria monocytogenes*; industrial surfaces; bacteriocin; *Lactobacillus sakei* CRL1862

INTRODUCTION

Adhesion of pathogenic and/or spoilage microorganisms to equipment materials and biofilm development constitute a potential chronic source of microbial contamination threatening the safety and quality of food products, resulting in food-borne disease and economic losses. Microbial adhesion to surfaces and consequent biofilm formation have been widely documented in different environments, particularly in meat and dairy processing plants (Simões, Simões and Vieira 2010). Abundant evidences indicate that biofilm mode of life leads to increased resistance to sanitizers when compared to planktonic cells, making their elimination a huge challenge (Davidson and Harrison 2002). Effort has been put forth to deepen the knowledge on microbial biofilms, defined as complex microbial communities established on a wide range of surfaces and embedded in a

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Table 1. *Listeria* strains used in this study.

Strain		Source	Serotype
Listeria innocua	7	Food (INRA, France)	6b
Listeria innocua	11	Food (INRA, France)	6b
Listeria innocua	12	Food (INRA, France)	6b
Listeria ivanovii	L1PE	Food (INRA, France)	5b
Listeria monocytogenes	FBUNT	Clinical (FBUNT, Argentine)	4b
Listeria monocytogenes	Scott A	Clinical (IHT, Karlsruhe-Germany)	4b

self-produced matrix of extracellular polymeric substances (Abee *et al.* 2011).

Listeria species are ubiquitous; however, only two species, *L. monocytogenes* and *L. ivanovii* are pathogenic for human and ruminants. *Listeria monocytogenes* demonstrated a remarkable ability to colonize and persist on food-processing environments including refrigerated premises (Bridier *et al.* 2015). However, *Listeria* strains were variable in their ability to produce biofilms, largely depending on serotype, lineage, origin of isolation, nutrients presence and temperature (Kadam *et al.* 2013). Increasing interest in identifying safe and effective antimicrobial approaches for controlling *L. monocytogenes* biofilms in food premises has been perceived. Different strategies to control biofilms have emerged, exploration of eco-friendly biocides *in-situ* produced representing a promising approach. Lactic acid bacteria (LAB) were reported to be good candidates to settle protective positive biofilms on food industry surfaces playing a key role in controlling *L. monocytogenes* colonization (Simões, Simões and Vieira 2010; Winkelströter *et al.* 2011; Ndahetuye *et al.* 2012). LAB and their bacteriocins have been well documented for their antimicrobial activity against *L. monocytogenes* (Gálvez *et al.* 2007). Recently, meat-borne *Lactobacillus sakei* strains harboring bacteriocins-encoding genes were reported as highly effective at inhibiting *Listeria* strains (Fontana *et al.* 2015); the ability of these strains to grow in biofilm on abiotic surfaces at low temperatures was also described (Pérez Ibarreche, Castellano and Vignolo 2014). The aim of this study was to evaluate the ability of *L. sakei* CRL1862 and its bacteriocin to compete with, exclude and displace *L. monocytogenes* FBUNT during biofilm formation on industrial surfaces at 10°C.

MATERIALS AND METHODS

Bacterial strains and cultivation

Lactobacillus sakei CRL1862 from artisanal sausages (CERELA, culture collection) was cultured in MRS broth at 30°C for 18 h. *Listeria* strains (Table 1), were grown in BHI broth at 30°C for 16–18 h. In addition, other media were used for microtiter assays: TSB, modified TSB (mTSB) added with 2% of proteose peptone and modified Luria Bertani (mLB) containing per liter: 10 g NaCl, 5 g yeast extract, 10 g meat peptone. Unless otherwise stated, all the media used were supplied by Britania laboratory (Buenos Aires, Argentina).

Listeria strains serotyping and *in vitro* biofilm assay

Strains (Table 1) characterization was achieved by classical serotyping method (Seeliger and Höhne 1979).

Biofilm formation was investigated by the crystal violet staining method as described by Pérez Ibarreche *et al.* (2014). Briefly, overnight cultures from BHI broth were used as inoculum (5% v/v) and incubated during 24, 48 and 72 h at 30°C in a 96 wells microtiter plates. OD_{570} values from the different conditions above the cut-off line were considered positive for biofilm formation. Each strain and/or condition was tested in at least three independent experiments, each with four biological replicates.

Cell surface properties of selected biofilmogenic *Listeria* strain

Hydrophobic/hydrophilic, Lewis acid-base and auto-aggregation characteristics were investigated according to Pérez Ibarreche *et al.* (2014). Each measurement was performed in triplicate and the experiment was repeated twice with independent bacterial cultures.

Bacteriocin production by *L. sakei* CRL1862

Lactobacillus sakei CRL1862 (1% v/v) was inoculated in MRS broth (one liter) and incubated at 30°C for 18 h. Cells were removed by centrifugation, supernatant pH was adjusted to 6.5 (5N NaOH) and saturated with ammonium sulfate (60%). After 30 min stirring at 4°C the bacteriocin was pelleted by centrifugation and dissolved in 20 mM phosphate buffer (pH 6.5). Three milliliters of cells free supernatant were loaded onto a Sep-Pak C-18 cartridge (500 mg, Agilent, USA). Peptides were eluted with solvent A (0.1% trifluoroacetic acid in water) and solvent B (0.1% trifluoroacetic acid in acetonitrile). Each fraction was concentrated under vacuum and immediately assayed for inhibitory activity against *L. monocytogenes* FBUNT by agar diffusion test and the AU mL^{-1} were calculated (Castellano *et al.* 2010).

Surfaces and cleaning treatment

Stainless steel (SS) and polytetrafluoroethylene (PTFE) were used as matrix for biofilm formation and were conditioned as described by Pérez Ibarreche *et al.* (2014). Briefly, before using, chips were soaked with acetone, rinsed with distilled water and then soaked again with 1N NaOH. A final rinse with distilled water was carried out and SS and PTFE chips were air dried.

Control of *L. monocytogenes* FBUNT biofilm on SS and PTFE chips by the bacteriocin-producing *L. sakei* CRL1862

The ability of *L. sakei* CRL1862 to inhibit pathogenic biofilm was evaluated by displacement, exclusion and competition, schematic representation is shown in Fig. 1. For the

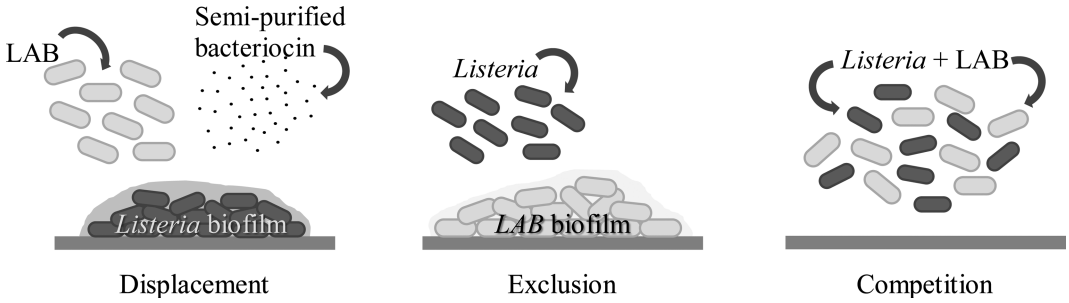

Figure 1. Scheme of the inhibition strategies of *Listeria* by *L. sakei* CRL1862 and/or its bacteriocin. Light gray rods represent *L. sakei* CRL1862 cells, while gray rods correspond to *Listeria* cells while small points represent semi-purified bacteriocin solution. 'Displacement': *L. sakei* CRL1862 cells and/or semi-purified bacteriocin solution were added to previously formed *Listeria* biofilm. 'Exclusion': *Listeria* cells were added to previously formed *L. sakei* CRL1862 biofilm. 'Competition': planktonic *L. sakei* CRL1862 and *Listeria* cells were co-cultured on the chip surfaces.

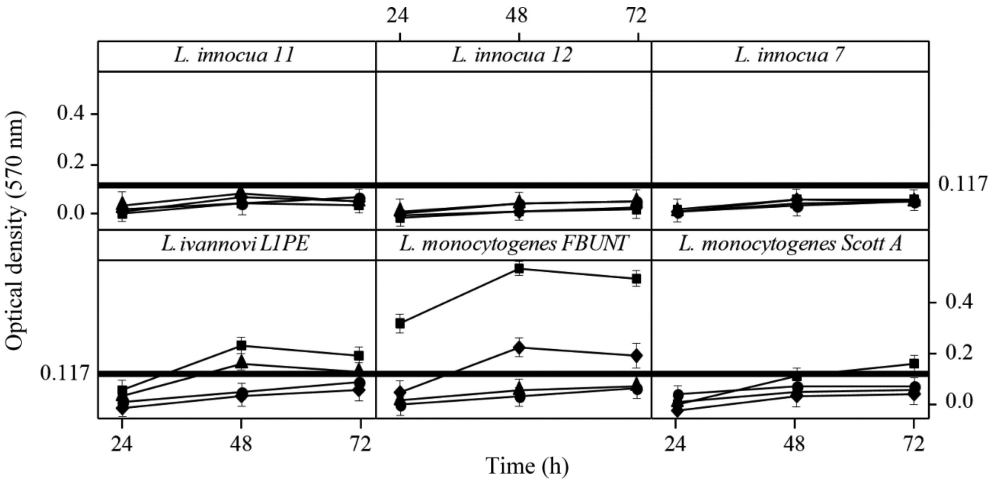

Figure 2. Scatterplot analysis for biofilm formation by *Listeria* strains evaluated at 30°C during 72 h and incubated in mLB (filled square); mTSB (filled diamond); TSB (filled triangle) and BHI (filled circle). Cut off line (solid black line).

'displacement' assay, *L. monocytogenes* FBUNT biofilm was initially formed on both surfaces (SS and PTFE) by incubation in diluted mLB (1:10) at 10°C during 6 days. Then, non-adherent cells were removed from the chips by rinse with PBS. *Lactobacillus sakei* CRL1862 cells (10^8 CFU mL^{-1}) and the semi-purified bacteriocin solution with 266.67 UA mL^{-1} (800 μL, each) were separately added to the previously prepared *L. monocytogenes* FBUNT biofilm (10^7–10^8 CFU mL^{-1}) and incubated for 6 h at 10°C. For the 'exclusion' assay, *L. sakei* CRL1862 biofilm was initially settled on both surfaces by incubation in MRS at 10°C during 6 days. Then, chips rinsed with PBS to remove non-adherent cells were immediately inoculated with *L. monocytogenes* FBUNT and incubated in diluted mLB for 2 and 4 days at 10°C. For the 'competition' assay, *L. sakei* CRL1862 and *L. monocytogenes* FBUNT were co-cultured aerobically at ~10^8 CFU mL^{-1} on SS and PTFE chips in diluted mLB at 10°C for 6 days. Cultures of *L. monocytogenes* FBUNT were used as controls. For enumeration, *L. monocytogenes* FBUNT biofilm cells were collected from the chips by rinsing and dispersed in 100 mL of PBS containing glass beads (6 mm) by vigorous agitation (10 min). Proper dilutions were plated on PALCAM agar (BD/Difco Laboratories Inc., Detroit, MI, USA) and incubated at 30°C for 48 h. Results were calculated as CFU of adherent cells per cm^2. Each measurement was performed in triplicate and repeated twice with independent bacterial cultures. Means and standard deviations were calculated. Significant differences ($P \leq 0.05$) between means were determined using ANOVA followed by Tukey test by using the InfoStat program (InfoStat Group, FCA, National University of Córdoba, Argentina, 2009).

RESULT AND DISCUSSION

Screening and selection of *Listeria* strain for biofilm formation

Results showed that the growth conditions and incubation time affected *Listeria* biofilm formed (Fig. 2). Although no biofilm formation was registered for *L. monocytogenes* Scott A and *L. innocua* strains, a high biofilm development was exhibited by *L. monocytogenes* FBUNT and *L. ivanovii* L1PE in nutrient-poor medium mLB, biofilm production being maximal at 48 h of incubation. The effect of serotype and isolation origin was not significant; *L. monocytogenes* strains (FBUNT and Scott A) of clinical origin and belonging to 4b serotype differed widely in biofilm production, irrespective of the medium used (Fig. 2; Table 1). Biofilm forming ability has been reported to be variable, independently of the origin of isolation, nutrient levels and serotype (Harvey, Keenan and Gilmour 2007; Nyenje, Green and Ndip 2012). Particularly for *L. monocytogenes*, differences in the ability to adhere to surfaces and significant differences for cell numbers after 24 h of biofilm growth were found (Harvey *et al.* 2007; Kadam *et al.* 2013).

Table 2. Hydrophobicity and auto-aggregation of *L. monocytogenes* FBUNT.

	Hydrophobicity (%)			
Growth medium	Hexadecane	Chloroform	Ethyl acetate	Auto-aggregation (%)
mTSB	13.8 ± 3.1	37.3 ± 4.3	8.8 ± 3.0	5.7 ± 0.5
mLB	49.2 ± 10.1	89.9 ± 2.0	20.6 ± 3.5	14.3 ± 2.0

Surface properties of selected biofilmogenic *L. monocytogenes* FBUNT

Adhesion ability of *Listeria* strains to polystyrene microtiter plates allowed selecting *L. monocytogenes* FBUNT as the higher biofilm producer. When superficial characteristics were investigated, adhesion to hexadecane was higher in mLB (49.2%) than in mTSB (13.8%) (Table 2), thus a moderate hydrophobic and hydrophilic surface character, respectively, may be inferred. Similar results were reported for *L. monocytogenes* epidemic strains (Chae *et al.* 2006). Moreover, the pathogen displayed higher adhesion to chloroform (37.3% and 89.9%) than to ethyl acetate (8.8% and 20.6%) in mTSB and mLB, respectively, indicating basic and electron-donor characteristics. Similarly, auto-aggregation showed higher values in cells grown in mLB medium (14.3%) compared with mTSB (5.7%). These results are in coincidence with those previously reported for *L. monocytogenes* strains of different origin by Miladi *et al.* (2013) and Woo and Ahn (2013).

Lactobacillus sakei CRL1862-mediated inhibition of biofilm formation by *L. monocytogenes* FBUNT on industrial surfaces

The biofilm formation by the meat-borne *L. sakei* CRL1862 on materials used in the meat industry under different conditions was described by Pérez Ibarreche *et al.* (2014). Besides this ability, this strain was found to carry the *sap*A structural gene encoding for curvacin A, an antilisterial bacteriocin (Fontana *et al.* 2015). On this basis, the inhibitory effect of *L. sakei* CRL1862 on biofilm formation by the selected *L. monocytogenes* FBUNT was evaluated in displacement, exclusion and competition on SS and PTFE chips during different times at 10°C (Fig. 3).

Displacement

Figure 3a shows the decrease of *L. monocytogenes* FBUNT sessile cells on SS and PTFE after the addition of bacteriocin-producing *L. sakei* CRL1862 planktonic cells and its semi-purified bacteriocin (266.67 AU mL^{-1}). *Lactobacillus sakei* planktonic cells as well as bacteriocin treatment were able to displace the pre-established pathogen biofilm by 2.22 and 1.77 log CFU cm^{-2} on SS and PTFE, respectively after 6 h. Similarly, reductions of *L. monocytogenes* pre-existing biofilm by a nisin-producing *Lactococcus lactis* and *Enterococcus durans* (Zhao *et al.* 2013) and probiotic strains (Woo and Ahn 2013) on different surfaces and temperatures, were reported. However, an enhanced pathogen inhibition was observed when the semi-purified bacteriocin extract was added, reductions of 3.09 and 3.56 log CFU cm^{-2} on SS and PTFE respectively occurred. This result was consistent with the production of the antilisterial bacteriocin (curvacin A) whose higher concentration in the extract was responsible for the more efficient antimicrobial effect. Even when inhibitory metabolites other than bacteriocin must have been produced by *L. sakei* CRL1862 during growth, inhibition of pre-established pathogen biofilm was less effective compared to the addition of the bacteriocin extract. Conversely, the combination of the antimicrobials produced by *L. lactis* UQ2 (lactic acid and nisin) was more efficient to suppress *L. monocytogenes* growth than the bacteriocins on its own (García-Almendárez *et al.* 2008).

Exclusion

Biofilm formation by *L. monocytogenes* FBUNT was prevented by a previously formed *L. sakei* CRL1862 biofilm on the abiotic surfaces, sessile cells decreased by 2.67 and 4.06 log CFU cm^{-2} on SS and PTFE after 96 h, respectively (Fig. 3b). The production of growth-inhibiting factors by *L. sakei* CRL1862 may have influenced the survival and multiplication of the pathogen in the biofilm. Similarly, high antilisterial efficacy was achieved by pre-established biofilms of bacteriocin-producers LAB as reported by Guerrieri *et al.* (2009) and Ndahetuye *et al.* (2012).

Competition

The ability of *L. sakei* CRL1862 planktonic cells to competitively inhibit biofilm formation by *L. monocytogenes* FBUNT was also investigated (Fig. 3c). During co-culture, pathogen sessile cells were decreased by 4.52 and 5.54 log CFU cm^{-2} after 6 days on SS and PTFE, respectively. Similarly, co-cultivation of bacteriocin-producing LAB strains showed to influence *L. monocytogenes* adhesion on SS surface (Ratti *et al.* 2010; Winkelströter *et al.* 2011). The production of inhibitory compounds (bacteriocins, organic acids, biosurfactants and enzymes) may delay/inhibit the growth of, prevent attachment of other species or even provoke detachment of cells from the biofilm structures (Rendueles and Ghigo 2012). In addition, the anti-adhesive effect of EPS produced by *L. sakei* CRL1862 would play a role in preventing the adhesion of *L. monocytogenes*, modifying the balance between biofilm and planktonic populations by diverting pathogen cells from the biofilm to the bulk medium (Leriche and Carpentier 2000; Pérez Ibarreche *et al.* 2014).

Different inhibition extents were observed among tested strategies; biofilm formation by *L. monocytogenes* was less inhibited in displacement assay than in competition and exclusion. This observation was consistent with the previous report that LAB-mediated effect was less effective in post- than pre-treatments (Tahmourespour, Salehi and Kermanshahi 2011; Woo and Ahn 2013). Depending on the composition of the chips as the matrix for biofilm development, the biofilmogenic ability of *L. monocytogenes* was variable; its inhibition on the hydrophilic surface (SS) was higher than on hydrophobic PTFE surface in displacement assay, in contrast to that observed during exclusion and competition. Zhao *et al.* (2013) reported that bacteriocins-producing LAB used as competitive exclusion bacteria reduce sessile *L. monocytogenes* cells pre-existing on chips composed of different materials at low temperatures, likely by producing bactericidal metabolites that kill the pathogen. Indeed, the amphiphilic nature of the bacteriocin produced by *L. sakei* CRL1862 (Daeschel, Mcguire and Al-Makhlafi 1992) on the abiotic surfaces would allow its adsorption retaining its activity on both surfaces. However, general experimental findings indicate that the

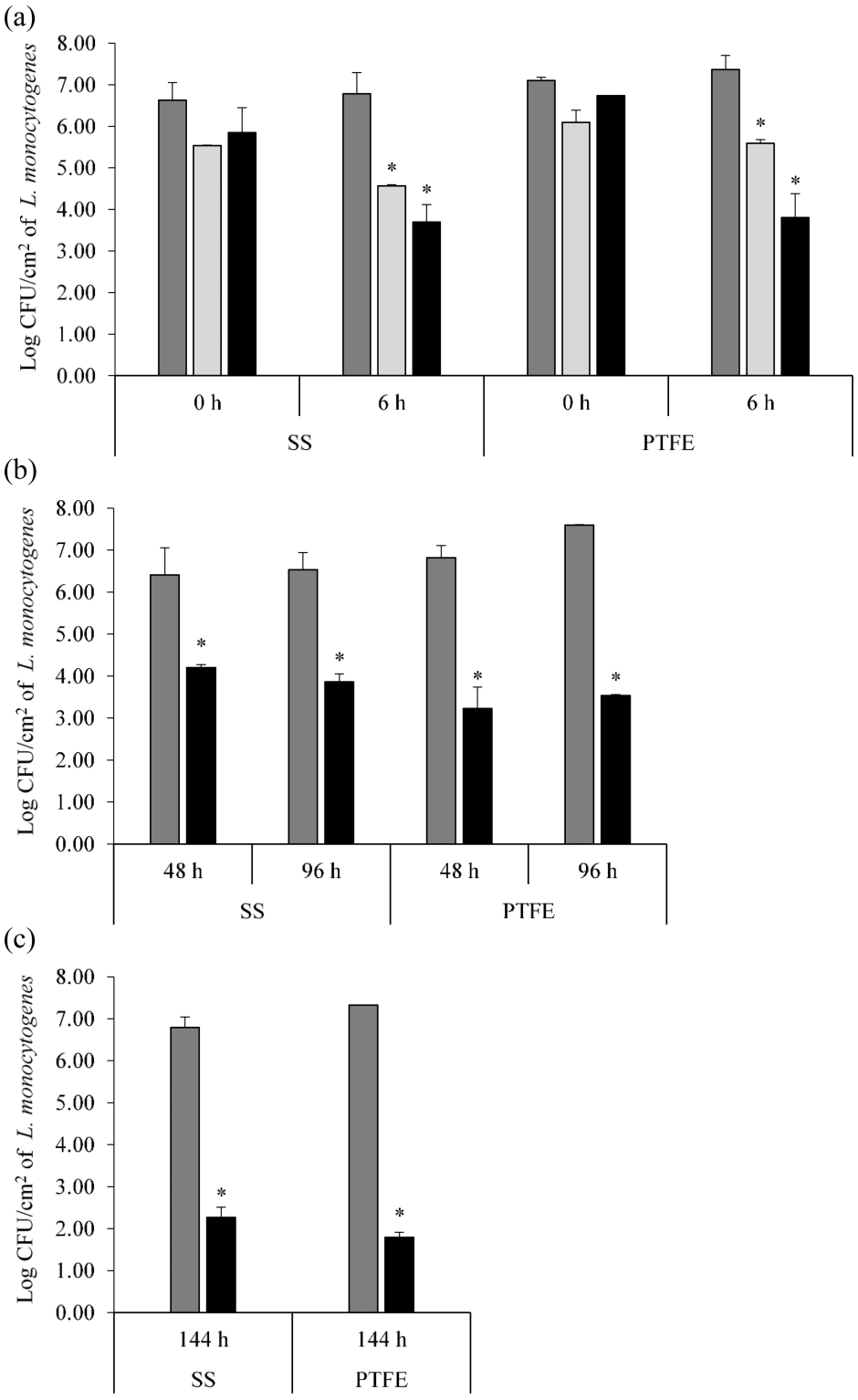

Figure 3. Control strategies for the inhibition of *L. monocytogenes* FBUNT biofilm on stainless steel (SS) and polytetrafluoroethylene (PTFE) chips by the bacteriocinogenic *L. sakei* CRL1862 and its partially purified bacteriocin at 10°C. (**a**) *Listeria monocytogenes* FBUNT numbers after displacement with *L. sakei* CRL1862 planktonic cells (light-gray bar), semi-purified bacteriocin extract (black bar) and control (dark-gray bar); (**b**) *L. monocytogenes* FBUNT numbers after exclusion by a pre-established *L. sakei* CRL1862 biofilm, treatment (black bar) and control (dark-gray bar) and (**c**) *L. monocytogenes* FBUNT numbers in competition (co-culture) with *L. sakei* CRL1862, treatment (black bar) and control (dark-gray bar). Asterisk indicates significant differences in the pathogen growth between controls and treated samples ($P < 0.05$).

affinity of proteins to surfaces increases on hydrophobic substrates and decreases on hydrophilic surfaces (Rabe, Verdes and Seeger 2011).

In conclusion, the adhesion reduction of *L. monocytogenes* on abiotic matrices can be a potential approach to mitigate contamination of industrial premises preventing pathogenic infections. Indeed, bacteriocinogenic *L. sakei* CRL1862 through the assayed strategies was able to reduce *L. monocytogenes* FBUNT biofilm on SS and PTFE surfaces. However, from an industrial point of view, displacement and exclusion would represent more realistic approaches.

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Conflict of interest. None declared.

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