1 ***Role of Lactobacillus* biofilms in *Listeria monocytogenes* adhesion to glass**
2 **surfaces**

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4 Abstract (252 words)

Listeria monocytogenes can form long-lasting biofilms on food-contact surfaces. Lactic acid bacteria (LAB) have shown promise in antagonizing this microorganism in liquid media. However, the ecological relationships differ when cells are forming biofilms. In this work, we propose the use of *Lactobacillus* biofilms as surface "conditioners" to modulate the adhesion of *L. monocytogenes*. For this, the biofilm formation ability of *Lactobacillus fermentum* MP26 and *Lactobacillus salivarius* MP14 (human milk origin), fluorescently labelled by transfer of the mCherry-encoding pRCR12 plasmid, was first evaluated. Then, mature biofilms of these strains transformed with pRCR12 for expressing the fluorescent protein mCherry were used as adhesion substrate for GFP-tagged *L. monocytogenes* Scott A. The resulting biofilms were studied in terms of cellular population and attached biomass (cells plus matrix). Species distribution inside

the biofilm structure was revealed by confocal laser scanning microscopy (CLSM). Although none of the *Lactobacillus* spp. strains reduced the adhesion of *L. monocytogenes* Scott A, species interactions seem to interfere with the synthesis of extracellular polymeric substances and species distribution inside the biofilms. In dual-species biofilms, CLSM images revealed that *Lactobacillus* cells were trapping those of *L. monocytogenes* Scott A. When surfaces were conditioned with *Lactobacillus* biofilms, the spatial distribution of *L. monocytogenes* Scott A cells was species-specific, suggesting these interactions are governing the ultimate biofilm structure. The results here obtained open new possibilities for controlling *L. monocytogenes* dispersal using these *Lactobacillus* spp. biofilms as a “natural” immobilization way. Whether species interactions could modify the virulence of *L. monocytogenes* still remains unclear.

Keywords: *Listeria monocytogenes*; biofilms; food safety; *Lactobacillus fermentum*; *Lactobacillus salivarius*; LAB; CLSM

1. Introduction

Biofilms are aggregates of microorganisms in which cells that are frequently embedded within a self-produced matrix of Extracellular Polymeric Substances (EPS) adhere to each other and/or to a surface (Vert et al. 2012). As part of these structures, microorganisms are more resistant to a variety of environmental conditions and treatments than cells in suspension (Flemming et al. 2016; Kim et al. 2018). The high cellular density and the close contact between cells increase the probability of genetic transfer, including antibiotic resistance genes, and favor the sharing of metabolites that can benefit some members of the community (Bridier et al. 2011; Verraes et al. 2013; Xavier et al. 2005;). Indeed, recent studies suggest that isolated bacteria that would have little chance of persisting in hostile environments, can do it when “hosted” in biofilms (Puga et al. 2018; Xavier et al. 2005). Hence, their presence in clinical and food settings is considered a potential health risk (Brooks and Flint, 2008; Kumar and Anand, 1998; Lindsay and von Holy, 2006; Otter et al. 2015).

In food processing plants, the Hazard Analysis and Critical Control Point system (HACCP) together with the implantation of strict cleaning and disinfection protocols are essential to preserve food safety (Choi et al. 2019; Holah, 1995). However, the accessibility of certain parts of the equipment that is in contact with food may be limited, making difficult their maintenance in a proper sanitation state. In addition, the doses and application times of disinfectants are usually adapted for killing planktonic microorganisms, but they may be inefficient when fighting against biofilms (Coughlan et al. 2016). In cutters, conveyor belts, drains, walls and ceilings, the risk of biofilm formation is particularly high, rising the risk of microbial transfer to food (Carpentier and Cerf, 2011; Gandhi and Chikindas, 2007; Griffith, 2005; Moretro and Langsrud, 2017).

The presence of *Listeria monocytogenes* on food-contact surfaces is particularly concerning due to its extraordinary resilience. This microorganism causes listeriosis, a low-incidence disease but with the highest mortality rate among foodborne zoonoses, leading to a relevant medical, social and economic burden (EFSA, 2018). Moreover, it is the first cause of outbreaks related to the consumption of contaminated Ready-To-Eat food (Zhao et al. 2004). There are several factors that make *L. monocytogenes* so elusive to control measures including its ability to persist in food processing plants for months or years, even at refrigeration temperatures (Martínez-Suárez et al. 2016; Orgaz et al. 2013; Valderrama and Cutter, 2013), its ability to form mono- or multispecies biofilms (Guerrieri et al. 2009; Puga et al. 2016a), or its ability to integrate into biofilms previously developed by other species, such as *Pseudomonas fluorescens* (Puga et al. 2018). As a consequence, *L. monocytogenes* can overcome some of the classical hurdles used in the food industry for controlling microbial proliferation (e.g. low temperature and disinfectants), being necessary to search for alternative approaches to control this microorganism.

Numerous studies have shown that *L. monocytogenes* population can be reduced, inhibited or displaced when growing in mixed cultures with lactic acid bacteria (LAB),

fundamentally belonging to the genera *Lactococcus* and *Lactobacillus* (Gómez et al. 2016; Leriche et al. 1999). Recently, Cruciata et al. (2018) demonstrated that the presence of LAB biofilms on the surface of wooden vats intended for the production of traditional Sicilian cheeses inhibited the adhesion of *L. monocytogenes* to these surfaces. LAB may antagonize the growth of pathogenic microbes (Bambirra et al. 2007; Gómez et al. 2016; Hossain et al. 2017; Woo and Ahn, 2013) by several mechanisms, such as the production of bacteriocins, organic acids and other antimicrobial compounds, the production of surfactants and, also, via spatial and nutrient competition (Cani et al. 2009; Guilloteau et al. 2010; Hancock et al. 2010; Kanmani et al. 2013). However, with a few exceptions, most of these antagonism studies were performed with microorganisms growing planktonically and the ecological relationships observed in liquid cultures can differ when cells are forming biofilms (Foster and Bell, 2012; Nadell et al. 2009). In fact, competing species can be found sharing the same biofilm and forming rather stable microbial communities in real environments (Giaouris et al. 2015).

In terms of spatial microbial competition it is important to consider which species arrives first at a surface, as first colonizers can occupy a privileged position inside a biofilm. Taking that into consideration, in this work we propose the use of *Lactobacillus* biofilms as surface "conditioners" in order to modulate the adhesion of *L. monocytogenes*. Initially, the biofilm formation ability of two *Lactobacillus* strains (*Lb. fermentum* MP26 and *Lb. salivarius* MP14), which had been isolated from human milk in a previous work, was tested in glass surfaces. Then, both strains were fluorescently labeled by transfer of the pRCR12 plasmid (Russo et al. 2015), which encodes the mCherry red fluorescent protein, and their biofilm structure was visualized by confocal laser scanning microscopy (CLSM). Finally, mature biofilms of these strains were used as adhesion substrate for green fluorescent protein (GFP)-tagged *L. monocytogenes* Scott A carrying plasmid pLS1ROM-GFP (Garay-Novillo et al. 2019; Puga et al. 2018;

Ruiz-Masó et al. 2012). The resulting biofilms were studied in terms of cellular population, attached biomass (cells plus matrix) and species distribution inside the biofilm structure.

2. Materials and Methods

2.1. Bacterial strains and growth conditions.

Two *Lactobacillus* strains, *Lb. fermentum* MP26 and *Lb. salivarius* MP14, both isolated from human milk, and *L. monocytogenes* Scott A (serotype 4b, lineage I) were used in this work. The human milk samples from which the *Lactobacillus* strains were isolated were collected in the frame of a previous study (Martín et al. 2012). Proper peptone water dilutions of the samples were plated onto De Man Rogosa and Sharpe (MRS, Oxoid; Basingstoke, UK) agar plates supplemented with L-cysteine (0.5 g/L) (MRS-Cys) (Sigma; St. Louis, Missouri, USA) which were incubated anaerobically (85% nitrogen, 10% hydrogen, 5% carbon dioxide) in a anaerobic workstation (MINI-MACS, DW Scientific, Shipley, UK) at 37°C for 48 h. At least one representative of each colony morphology was selected from the agar plates. Among them, two isolates (MP26 and MP14) were selected for the present study because of the following properties: (a) they displayed the fastest ability to form biofilms on glass surfaces in preliminary studies; and (b) they showed good growth ($\sim 9 \log_{10}$ cfu/mL) after aerobic incubation in MRS broth at 37°C for 16 h. Subsequently, they were identified as *Lb. fermentum* MP26 and *Lb. salivarius* MP14 by 16S rRNA gene sequencing as described previously (Kullen et al. 2000). Both species has the Qualified Presumption of Safety according to the European Food Safety Authority (EFSA). For cultivation, isolated colonies of both *Lactobacillus* strains grown in MRS-Cys and isolated colonies of *L. monocytogenes* Scott A grown in PALCAM (Oxoid) were transferred to 10 mL of MRS broth or trypticase soy broth (TSB, Oxoid), respectively, and incubated overnight at 37 °C. Then, cells were recovered by centrifugation at 17,000xg for 10 min at 4 °C, washed twice and suspended into fresh medium. The optical density at 600 nm (OD_{600}) of the

cellular suspensions was adjusted to a value of 0.15, corresponding to a cellular density of approximately 10^7 cfu/mL. When required, erythromycin (Sigma) or chloramphenicol (Sigma) were added at a final concentration of 5 µg/mL and 10 µg/mL, respectively.

For the fluorescent tagging of *Lactobacillus* cells, plasmid pRCR12, which constitutively expresses the *mrfp* gene encoding red fluorescent protein mCherry (Russo et al. 2015), was transferred to both *Lb. fermentum* MP26 and *Lb. salivarius* MP14 following the protocol described by (Berthier et al. 1996). Briefly, electrocompetent cells were prepared by centrifugation of stationary phase cultures obtained at 30 °C in MRS supplemented with 1% sucrose. The cell pellet was washed twice with 10 mL of ice-cold 10 mM MgCl₂ and suspended in 0.5 mL of 0.5 M sucrose/10% (v/v) glycerol. For plasmid transfer, pRCR12 (0.5 µg) was mixed with 50 µL of the competent cells in a 0.2-cm cuvette, and the conditions of electroporation were 1.8 kV, 600 Ω, and 25 µF. Transformants were selected on MRS-agar plates supplemented with 10 µg/mL chloramphenicol. GFP-tagged *L. monocytogenes* Scott A was obtained in a previous work (Puga et al. 2018) using plasmid pLS1ROM–GFP (Ruiz-Masó et al. 2012) for transformation. In this case, the transformed strain was cultured on TSB plates supplemented with 5 µg/mL erythromycin from a stock culture kept at –80 °C in TSB supplemented with 15% (v/v) glycerol.

2.2. Experimental system for biofilm growth.

Biofilms were developed at 37 °C on commercial 22×22 mm, microscope glass coverslips, using a batch system called carousel as described by (Orgaz et al. 2011). Briefly, sixteen coverslips were inserted vertically into the narrow radial slits of a Teflon platform (6.6 cm diameter). The platform and its lid were assembled by an axial metallic rod for handling and placed into a 600 mL beaker (Figure S1). The whole system was heat-sterilized as a unit. Sixty mL of the culture medium were inoculated with 1 mL of the previously obtained cellular suspensions to achieve an initial

concentration of 10^5 cfu/mL. For dual-species biofilms, microorganisms were inoculated to give a 1:1 ratio of both strains. For surface conditioning experiments, 24 h *Lactobacillus* biofilms were first developed into the coverslips surfaces. Then, they were washed to remove weakly attached cells by immersion of the whole carousel platform into a beaker containing 100 mL of sterile 0.85% (w/v) saline. This step was repeated twice. The platform was finally introduced into a new beaker containing 60 mL of medium inoculated with *L. monocytogenes* Scott A [pLS1ROM-GFP] and incubated for 72 h statically at 37 °C. Under these conditions, biofilm growth covered approximately 70% of the coverslip's surface, having a submerged area and a liquid-air interphase.

2.3. Cell recovery and counting.

At each extraction time, two diametrically opposed coupons were aseptically withdrawn from the platform and washed into 0.85% (w/v) saline to remove weakly attached cells. Both sides of the coverslip surface were thoroughly scraped using a sterile swab that was introduced into 1.5 mL of peptone water and vortexed at high speed to release cells. Decimally diluted samples were plated onto MRS-Cys and PALCAM and incubated at 37 °C for 24 h for counting of *Lactobacillus* and *L. monocytogenes* colony forming units, respectively.

2.4. Biomass determination.

For the quantification of the attached biomass (cells plus matrix), biofilms were stained for 2 min with a Coomassie Blue (Brilliant Blue R, Sigma) solution in acetic acid/methanol/distilled water (1:2.5:6.5). This step was repeated twice. Colorant excess was removed with water and coupons were air dried. These samples were afterwards immersed into 4 mL of the above mentioned solvent mixture (without the colorant) and the whole biomass was detached with sterile cell scrapers. After full homogenization of this suspension, OD was measured in a spectrophotometer using a wavelength of 595

nm. Bare coupons were stained the same way and used as controls. In parallel, images of the dried coupons were scanned using a HP Scanjet 300 at a resolution of 600 dpi.

2.5. Confocal Laser Scanning Microscopy (CLSM).

Biofilms images were acquired using a Nikon Ti-E inverted microscope equipped with a Nikon C2 confocal scanning module and an immersion objective at 63x. The excitation and emission wavelengths used for GFP and mCherry were 488 nm and 516 nm, respectively. 3D reconstructions from z-stacks (z step of 0.1 μ m) were carried out using IMARIS 8 software (Bitplane, Zürich, Switzerland). Biovolume quantification was performed using the MeasurementPro module of the above mentioned software. For this, three images of three independent coupons were analyzed.

2.6. Statistical analysis and data treatment.

Four independent experiments were performed to obtain mean values (n=4). Data were analyzed with Statgraphics Centurion software (Statistical Graphics Corporation, Rockville, Md., USA). One-way analysis of variance (ANOVA) was carried out to determine whether samples were different or not at a 95% confidence level (p<0.05).

3. Results

3.1. Effect of the presence of *Lb. fermentum* MP26 and *Lb. salivarius* MP14 on the adhesion capacity of *L. monocytogenes* Scott A.

Figure 1 shows the population of *L. monocytogenes* Scott A [pLS1ROM-GFP] (A) and *Lb. fermentum* MP26 [pRCR12] (B) over time in the three different types of biofilm, i.e. in monospecies, mixed biofilms (both species in the proportion of 1:1) and when *Lb. fermentum* MP26 biofilms were conditioning the surfaces. During the first 4 h, *L. monocytogenes* counts increased significantly (p<0.05) when growing in the presence of *Lb. fermentum*, particularly when surfaces were previously conditioned with this

microorganism. However, this trend was not maintained along the incubation period, being the attached population of *L. monocytogenes* significantly lower ($p<0.05$) in the presence of *Lb. fermentum* preformed biofilms (Fig. 1A), especially at the end of the incubation.

The presence of *L. monocytogenes* did not promote the early adhesion of *Lb. fermentum* (Fig. 1B); on the contrary, it had a significant detrimental effect when forming dual-species biofilms ($p<0.05$). The population of *Lb. fermentum* preformed biofilms was practically constant up to the 24 h of incubation, being reduced ($\sim 1 \log_{10}$) at the end of the incubation, when nutrient scarcity probably favored a dispersal phenomenon.

Assays performed with *Lb. salivarius* MP14 [pRCR12] showed a similar pattern (Fig. 2A). Presence of *Lb. salivarius*, both in mixed biofilms and when acting as surface's conditioner, fastened the initial adhesion (first 4 h) of *L. monocytogenes*. However, the decay of the *L. monocytogenes* population from these biofilm models appeared to be most effective when it grew in the presence of preformed biofilms of *Lb. salivarius*. In this case, *L. monocytogenes* counts decreased by $\sim 2 \log_{10}$ from 24 h to 72 h. As previously observed with *Lb. fermentum* MP26 [pRCR12], the presence of *L. monocytogenes* was whether neutral or detrimental for the adhesion of *Lb. salivarius* MP14 [pRCR12] (Fig. 2B).

3.2. Effect of the presence of *Lb. fermentum* MP26 and *Lb. salivarius* MP14 preformed biofilms on the biomass production.

In order to test if the surface conditioning modified not only the adhesion ability of *L. monocytogenes* Scott A but also the matrix production of both lactobacilli species when interplaying occurs, the total biomass of the biofilms was measured. This parameter includes both cells and extracellular matrix produced by the microorganisms. OD values of the three types of biofilm used in this work are shown in Figure 3. No

statistically significant differences ($p>0.05$) were found in OD values between *L. monocytogenes* Scott A [pLS1ROM-GFP] monospecies and mixed biofilms (1:1), independently of the accompanying *Lactobacillus* strain. In the case of *Lactobacillus* and specially at the end of the incubation (72 h) OD values of monospecies biofilms were significantly higher ($p<0.01$) than those of mixed biofilms (0.32 vs 0.1 and 0.26 vs 0.1 for *Lb. fermentum* and *Lb. salivarius*, respectively). Whether in monospecies or in mixed biofilms OD values remained very low (between 0.1-0.3) throughout the incubation period. However, when lactobacilli biofilms were conditioning the surfaces, OD values increased notably (between 3 and 5-fold of those achieved in the case of mixed biofilms), reaching a maximum between 24-48 h. Interestingly enough, in terms of cellular density at 24 h little or no statistically significant differences were observed between mixed and preformed biofilms, especially in the case of the *Lb. salivarius*-*L. monocytogenes* pair (Figs. 1 and 2).

A drastic reduction (almost 60% of the attached biomass was lost) at the end of the incubation was registered when *Lactobacillus* biofilms were conditioning the surfaces (Fig. 3). This downshift in OD values could be partly ascribed to cell detachment. As pointed previously, a concomitant reduction in *L. monocytogenes* and *Lactobacillus* populations was observed at the end of the incubation period when surfaces were conditioned with both *Lactobacillus* strains (Fig. 1 and 2).

3.3. Preformed *Lactobacillus* biofilms modify the surface colonization pattern of *L. monocytogenes* Scott A.

Images of the surface colonization pattern of the three different types of biofilms over time are shown in Figure 4. *L. monocytogenes* Scott A [pLS1ROM-GFP] showed a certain tendency to colonize clean surfaces more efficiently (Fig. 4A). A clear blue area, indicative of *L. monocytogenes* adhesion, appeared in the immersed area around 12 h incubation and the intensity of the air-liquid interphase increased after 24 h. A similar colonization pattern was observed when 1:1 biofilms were developed on clean

surfaces, independently of the *Lactobacillus* strain (Fig. 4C and 4F). When the coupons were first conditioned with *Lb. fermentum* MP26 [pRCR12] or *Lb. salivarius* MP14 [pRCR12] a rather dense biofilm (as indicated by intense blue staining) was observed from the beginning (1 h). It should be noted that this initial step corresponded to a 24 h mature biofilm of either *Lb. fermentum* or *Lb. salivarius* (Fig. 4D and 4G, respectively). Interestingly, samples of mixed biofilms withdrawn after 24 h (Fig. 4C and 4F) did not show such blue intensity despite the biofilm composition was rather similar in terms of cells (Fig. 1 and 2). Moreover, monospecies *Lactobacillus* biofilms OD values were on average higher than those of mixed biofilms (at least from 12-24 h incubation). This suggests that the presence of *L. monocytogenes* from the beginning of the cultivation could interfere with the matrix production of these *Lactobacillus* strains. Nevertheless, when *L. monocytogenes* found an already mature *Lactobacillus* biofilm, it was able to fully integrate into the structure, contributing to an increase in the measured biomass (Fig. 3). Moreover, in these samples the detachment of a large part of the biofilm (that of the immersed area) was observed after 24 h of incubation, as indicated by the sudden fall of the OD values (Fig. 3).

3.4. Species distribution inside the biofilm structure.

Both strains of *Lactobacillus* attached to coupon surfaces after 24 h incubation, although *Lb. fermentum* MP26 [pRCR12] surface colonization was more effective and homogeneous (Fig. 5A). On the contrary, *Lb. salivarius* MP14 [pRCR12] colonization was characterized by the presence of smaller cell clusters surrounded by areas with scattered cells all over the surface (Fig. 5B), similar to the colonization pattern showed by *L. monocytogenes* Scott A [pLS1ROM-GFP] (Fig. 5C). Figure 6 displays CLSM images of species distribution inside dual-species biofilms. The presence of *L. monocytogenes* clearly modified the colonization pattern of both *Lactobacillus* strains (Fig. 5). In the case of *Lb. fermentum* (Fig. 6.1), its ability to spread all over the surface was strongly reduced, favoring the formation of microcolonies. In these structures, *L.*

monocytogenes cells were preferentially located at the inner part and at the top whereas the bottom part of the microcolonies was totally occupied by cells of *Lb. fermentum* (Fig. 6.1.A and B). In the case of *Lb. salivarius*, the presence of *L. monocytogenes* had a more pronounced effect (Fig. 6.2) and, interestingly, the opposite colonization pattern was observed. *L. monocytogenes* cells were the first colonizers (Fig. 6.2.B) forming small aggregates at the inner part of the biofilm around which the *Lb. salivarius* cells were located. Figures 7.1 and 7.2 show *L. monocytogenes* distribution inside the structure of *Lb. fermentum* and *Lb. salivarius* preformed biofilms, respectively. In the first case, *L. monocytogenes* cells were located at the inner and at the top layers of the preformed biofilm. Nevertheless, its presence on the bottom layers of the *Lb. fermentum* structure was not observed, suggesting that microbial and/or physical exclusion may be occurring. On the contrary, *L. monocytogenes* cells were totally integrated into the preformed biofilm when *Lb. salivarius* was conditioning the surfaces, suggesting that in this case species interactions could be neutral or even positive.

4. Discussion

Food processing plants are privileged environments for microbial proliferation. Organic residues present in areas that are difficult to clean serve as nutrient source for in-plant microorganisms which can end up forming biofilms (Lamas et al. 2018). Once formed, their inhabitants can persist there for months or even years without being eliminated and, eventually, they can be transferred from surfaces to food (Carpentier and Cerf, 2011; Moretro and Langsrud, 2017). This is the case of *L. monocytogenes*, one of the most important pathogens in the food context (EFSA, 2018). One of the strategies proposed to minimize or inhibit the presence of this microorganism is the use of probiotic microorganisms, mainly LAB incorporated into daily cleaning solutions (Gómez et al. 2016). Given that species interactions differ when microorganisms are forming part of biofilms (Giaouris et al. 2015), the efficacy of these strategies, often

tested in liquid media, can be compromised. In this novel approach we wanted to test the impact of potentially probiotic biofilms on the modulation of *L. monocytogenes* Scott A adhesion to surfaces.

Lb. fermentum and *Lb. salivarius* are among the *Lactobacillus* species most frequently isolated from human milk (Fernández et al., 2013). The inhibitory effect of several strains of these two species on the growth of *L. monocytogenes* and other Gram-positive and Gram-negative pathogens has already been demonstrated *in vitro* and in animal studies (Chen et al. 2012; Klayraung and Okonogi, 2009; Olivares et al. 2006; Shin et al. 2012). Potentially probiotic microorganisms produce natural antimicrobial substances effective in inhibiting the growth of pathogens, such as biosurfactants (Englerova et al. 2018) or bacteriocins (Také et al. 2015). Moreover, various *Lactobacillus* strains have been shown to reduce the adhesion ability of *L. monocytogenes* to abiotic surfaces (Pérez Ibarreche et al. 2014; Son et al. 2016). However, there is no consensus on the role of LAB to prevent adhesion of *L. monocytogenes* to surfaces since other studies have shown that mixed biofilms of *Lb. plantarum* and *L. monocytogenes* were more resistant to different disinfectants than the corresponding monospecies, suggesting a potential transference of resistance genes between these species (van der Veen and Abee, 2011). Other studies indicated that various factors, including the production of exopolysaccharides by LAB strains, may positively or negatively affect the growth of different pathogens depending on the growth conditions (Kim et al. 2006). In our study, co-cultivation of *L. monocytogenes* Scott A with either *Lb. fermentum* MP26 or *Lb. salivarius* MP14 did not inhibit the adhesion ability of the former as the viable attached population of *L. monocytogenes* Scott A remained stable along the incubation (Figs. 1 and 2).

When *L. monocytogenes* Scott A was co-cultivated with both *Lactobacillus* strains, biofilm biomass did not increase with respect to monospecies biofilms (Figs. 3 and 4). Further, low OD values were obtained at 24 h in mixed biofilms compared with those of

L. monocytogenes plus preformed biofilms (0.1 vs. 0.6 and 0.1 vs. 0.9 in the case of *Lb. fermentum* MP26 and *Lb. salivarius* MP14, respectively) (Figs. 3A and B). This suggest that, although both microorganisms are capable of coexisting in dual-species biofilms, interspecies interactions could have interfered with the expression of genes related to EPS synthesis and/or adhesion ability. Transcription of *luxS* gene was up-regulated in *Lb. acidophilus* NCFM when co-cultivated with *L. monocytogenes* EGD-e, improving *Lb. acidophilus* NCFM adhesion capacity to Caco-2-cells (Moslehi-Jenabian et al. 2011). Our results did not show a long-term improvement of the adhesion ability of none of the two *Lactobacillus* strains tested in this work (Figs. 1B and 2B) although EPS production seems to be somehow impaired when both microorganisms start forming dual-species biofilms “de novo”. Moreover, CLSM images of dual-species biofilms after 24 h (Figs. 6.1 and 6.2) revealed the detrimental impact of the co-cultivation on the structure previously observed in monospecies biofilms (Fig. 5). Dual-species biofilms became thinner and *Lactobacillus* cells were preferentially surrounding those of *L. monocytogenes* instead of spreading all over the surface. Coaggregation of LAB with pathogenic microorganisms other than *L. monocytogenes* has been previously described in liquid cultures (Boris et al. 1998; García-Cayuela et al. 2014). Our results demonstrate that this phenomenon could be even more pronounced when microorganisms are in close contact forming biofilms.

When *Lb. fermentum* MP26 and *Lb. salivarius* MP14 biofilms were conditioning the surfaces, the early adhesion of *L. monocytogenes* Scott A improved compared to monospecies biofilms (Figs. 1A and 2A, respectively). In the absence of flow (as it is the case in this study), the early stages of surface colonization are dominated by weak hydrophobic interactions between the bacteria and the surface; such interactions become stronger later on, once microorganisms start synthesizing sticky extracellular polymers (Garrett et al. 2008). It must be highlighted that the adhesion substrate in this study was a mature biofilm instead of a hydrophilic material, such as glass coupons.

The extracellular matrix of *Lactobacillus* is mainly built up of exopolysaccharides of glucose, mannose or galactose residues (Xu et al. 2019), which could have helped *L. monocytogenes* planktonic cells to overcome initial repulsion forces and probably changed the adhesive properties of the surface. Also, preformed *Lactobacillus* biofilms far from being a passive surface act as an active source of metabolites that may have helped planktonic cells of *L. monocytogenes* to “sense” the surface. Recently, a process similar to a surface memory has been demonstrated in *Pseudomonas aeruginosa* via type IV pili (TFP) and cAMP signaling (Lee et al. 2018). According to the cited work, the repeated cycle of sensing, division and detachment creates a planktonic population better adapted for sensing and attachment, which may be especially relevant at early stages of biofilm formation.

CLSM images (Fig. 7.1) showed that when surfaces were conditioned with *Lb. fermentum* MP26 biofilms, *L. monocytogenes* Scott A cells were preferentially located at the inner layers of these structures after a 24 h incubation. This pattern has been previously documented in surfaces conditioned with *P. fluorescens* biofilms (Puga et al. 2018). *L. monocytogenes* is a facultative microorganism capable of forming biofilms under oxygen restrictive conditions (Lezzoum-Atek et al. 2019). The fact that *L. monocytogenes* Scott A migrates to the inner part of these structures may partly explain the observed decrease in its viable attached population at the end of the incubation (Fig. 1A). Cells there located have less access to oxygen and nutrients, and they could have enter into a viable but not cultivable state (Lewis, 2005). Therefore, the possibility that a part of the *L. monocytogenes* Scott A population was underestimated cannot be ruled out. On the other hand, an important part of the structure detached at the end of the incubation because of nutrient depletion (Fig. 3).

Competitive exclusion has been considered as one of the mechanisms by which LAB may displace potentially pathogenic species (Falagas and Makris, 2009). The results shown in this work do not support this idea, although species interactions seem to

interfere with EPS synthesis and species distribution inside the model biofilms employed in this study. Although the presence of the LAB strains did not reduce the adhesion of *L. monocytogenes* Scott A, the interactions between them may still be beneficial to improve the food safety; the results of this study revealed that the *L. monocytogenes* Scott A cells were trapped by the *Lactobacillus* microcolonies (Figs. 6 and 7) inhibiting its spreading all over the surface as in monospecies biofilms (Fig. 5C). This phenomenon may not only immobilize this pathogen but, also, lead to changes in the expression level of some of its virulence genes, reducing its pathogenicity. Work is in progress in order to elucidate such hypothesis. Although this work is a first step in a medium term strategy, by combining already existing technologies these *Lactobacillus* biofilms could be used to develop antimicrobial films (Chang et al. 2015; Yordshahi et al. 2020). In addition, human clinical trials have shown that strains belonging to these species are safe and efficient against infectious diseases (Maldonado et al. 2010; Maldonado et al. 2012; Maldonado-Lobón et al. 2015).

The fact that the resident microbiota living in the food processing plants might modify the ecological relationships here observed shouldn't be disregarded in order to improve this technology (Møretrø and Langsrud, 2017; Tousek et al. 2020).

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Figure captions

Figure 1. Attached population density over time of *L. monocytogenes* Scott A [pLS1ROM-GFP] **(A)** in: monospecies biofilms (white bars), dual-species biofilms (light grey bars), and over *Lb. fermentum* MP26 [pRCR12] preformed biofilms (dark grey bars). Attached population density over time of *Lb. fermentum* MP26 [pRCR12] **(B)** in:

monospecies biofilms (black bars), dual-species biofilms (light grey bars), and 24 h preformed *Lb. fermentum* MP26 [pRCR12] biofilms (dark grey bars). Error bars represent the standard deviation (n = 4). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure 2. Attached population density over time of *L. monocytogenes* Scott A [pLS1ROM-GFP] **(A)** in: monospecies biofilms (white bars), dual-species biofilms (light grey bars), and over *Lb. salivarius* MP14 [pRCR12] preformed biofilms (dark grey bars). Attached population density over time of *Lb. salivarius* MP14 [pRCR12] **(B)** in: monospecies biofilms (black bars), dual-species biofilms (light grey bars), and 24 h preformed *Lb. salivarius* MP14 [pRCR12] biofilms (dark grey bars). Error bars represent the standard deviation (n = 4). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure 3. Biomass (cells + matrix) evolution over time of *L. monocytogenes* Scott A [pLS1ROM-GFP] monospecies biofilms **(A and B)** (white bars), monospecies biofilms of *Lb. fermentum* MP26 [pRCR12] **(A)** and *Lb. salivarius* MP14 [pRCR12] **(B)** (black bars), dual-species biofilms with *Lb. fermentum* MP26 [pRCR12] **(A)** and *Lb. salivarius* MP14 [pRCR12] **(B)** (light grey bars), and over preformed biofilms of *Lb. fermentum* MP26 [pRCR12] **(A)** and *Lb. salivarius* MP14 [pRCR12] **(B)** (dark grey bars). Error bars represent the standard deviation (n = 4). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure 4. Surface colonization pattern of glass coupon surfaces over time by the different biofilms developed in this work. **(A):** *L. monocytogenes* Scott A [pLS1ROM-GFP]; **(B):** *Lb. fermentum* MP26 [pRCR12]; **(C):** *Lb. fermentum* MP26 [pRCR12]- *L. monocytogenes* Scott A [pLS1ROM-GFP] mixed biofilms; **(D):** *L. monocytogenes* Scott A [pLS1ROM-GFP] over 24 h biofilms of *Lb. fermentum* MP26 [pRCR12]; **(E):** *Lb. salivarius* MP14 [pRCR12]; **(F):** *Lb. salivarius* MP14 [pRCR12] - *L. monocytogenes*

Scott A [pLS1ROM-GFP] mixed biofilms, and **(G)**: *L. monocytogenes* Scott A [pLS1ROM-GFP] over 24 h biofilms of *Lb. salivarius* MP14 [pRCR12].

Figure 5. CLSM images of *Lb. fermentum* MP26 [pRCR12] **(A)**, *Lb. salivarius* MP14 [pRCR12] **(B)** and *L. monocytogenes* Scott A [pLS1ROM-GFP] **(C)** monospecies biofilms. Scale bar: 20 μ m.

Figure 6. CLSM images of *Lb. fermentum* MP26 [pRCR12] - *L. monocytogenes* Scott A [pLS1ROM-GFP] **(1)** and *Lb. salivarius* MP14 [pRCR12] - *L. monocytogenes* Scott A [pLS1ROM-GFP] **(2)** mixed biofilms. Lactobacilli cells are mCherry-tagged (in red) and *L. monocytogenes* cells are GFP-tagged (in green). On the right panel of each image, xz-view is divided into three slices (yellow plane) **(A)**: at the bottom, at the inner part and at the top of the biofilm. The corresponding zenital images of these planes are also displayed **(B)**. Scale bar: 20 μ m.

Figure 7. CLSM images of mCherry-tagged *Lb. fermentum* MP26 [pRCR12] **(1)** and *Lb. salivarius* MP14 [pRCR12] **(2)** preformed *biofilms* (in red) after 24 h inoculation with GFP-tagged *L. monocytogenes* Scott A [pLS1ROM-GFP] cells (in green). On the right panel of each image, xz-view is divided into three slices (yellow plane) **(A)**: at the bottom, at the inner part and at the top of the biofilm. The corresponding zenital images of these planes are also displayed **(B)**. Scale bar: 20 μ m.

Supporting information

Figure S1. Experimental system for biofilm development. Reprinted from Puga et al. (2016b), with permission from Elsevier.

Figure 2S. Log differences between species in mixed biofilms (MB) (1:1) and preformed biofilms (PB) with respect to their corresponding monospecies biofilms. Negative values indicate that the species population in these biofilms is lower than in

their monospecies counterparts. Log differences between *L. monocytogenes* Scott A [pLS1ROM-GFP] MB and its monospecies biofilms **(A and B)** (white bars); Log differences between *L. monocytogenes* Scott A [pLS1ROM-GFP] in PB and its monospecies biofilms **(A and B)** (grey bars); Log differences between *Lb. fermentum* MP26 [pRCR12] and *Lb. salivarius* MP14 [pRCR12] in MB and their monospecies biofilms **(A and B, respectively)** (light orange bars) and log differences between *Lb. fermentum* MP26 [pRCR12] and *Lb. salivarius* MP14 [pRCR12] in PB and their monospecies biofilms **(A and B, respectively)** (black bars).

Table 1S. *p*-values of the data displayed in figures 1, 2 and 3 corresponding to the following symbols: **p* < 0.05, ***p* < 0.01, ****p* < 0.001 (n=4).

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Table 1S. *p*-values of the data displayed in figures 1, 2 and 3 corresponding to: **p* < 0.05, ***p* < 0.01, ****p* < 0.001 (n=4).

Time (h)	Figure 1		Figure 2		Figure 3	
	A	B	A	B	A	B
1	0.0011/0.0011	0.0001	0.0045/0.0326	0.0098	0.0186/0.0006	0.0050
2	0.0046	0.0002	0.0075	0.0148	0.0023	0.0260/0.0020
3	0.0178	0.0133/0.0000	0.0376	0.0064	0.0201	0.0003
4	0.0008	0.0014/0.0001	0.0014/0.0046	0.0269/0.0019	0.0423	0.0694
8	0.1874	0.0119	0.0389	0.0046/0.0006	0.0417/0.0061	0.0018/0.0015/0.0007
12	0.1200	0.0016	0.6623	0.0026	0.0176	0.0002/0.0007
24	0.1029	0.0332	0.1038	0.1812	0.0009	0.0000
48	0.1029	0.0242	0.0065	0.5986	0.0002	0.0373/0.0012
72	0.3886	0.0254	0.0082/0.0004	0.7510	0.0084/0.0016/0.0128	0.0249/0.0063/0.0014

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 1S

Figure 2S