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# Evaluation of anti-*Listeria* meat borne *Lactobacillus* for biofilm formation on selected abiotic surfaces ☆

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ABSTRACT

The ability of meat borne anti-*Listeria Lactobacillus* to form biofilms under different in vitro conditions and on abiotic surfaces was investigated. Biofilm formation by the adhesion to polystyrene microtiter plates was determined, this being higher for *Lactobacillus curvatus* CRL1532 and CRL705 and *Lactobacillus sakei* CRL1862. The physicochemical properties of the cell surface were relatively hydrophilic and acidic in character; *L. sakei* CRL1862 exhibiting the strongest autoaggregation. The adhesion of lactobacilli to stainless steel (SS) and polytetrafluoroethylene (PTFE) supports at 10 °C was found to be maximal for *L. sakei* CRL1862 on SS after 6 days. When biofilm architecture was characterized by epifluorescence and SEM, *L. sakei* CRL1862 homogeneously covered the SS surface while cell clusters were observed on PTFE; the extracellular polymeric substance matrix adapted to the topography and hydrophilic/hydrophobic characteristics of each material. The feasibility of *L. sakei* CRL1862 to form biofilm on materials used in meat processing highlights its potential as a control strategy for *Listeria monocytogenes* biofilms.



## 1. Introduction

Microbial adhesion to solid surfaces and subsequent biofilm formation are major concerns in food, biotechnological, medical, marine and other industrial fields. In the food industry, adhesion of pathogenic and/or spoilage microorganisms to equipment materials and biofilm development is of great importance as a potential chronic source of microbial contamination threatening the safety and quality of food products, resulting in foodborne disease and economic losses (Carpentier & Cerf, 2011; Sofos & Geornaras, 2010). Microbial adhesion to surfaces and consequent biofilm formation have been documented in many different environments and particularly during poultry and meat processing (Simpson Beauchamp et al., 2012; Sofos & Geornaras, 2010; Somers & Wong, 2004). There is abundant evidence indicating that the biofilm mode of life leads to increased resistance to antimicrobial products (cleaners and disinfectants) when compared to planktonic cells, making their elimination from food facilities a big challenge (Somers & Wong, 2004).

Biofilms are described as cells bound together by extracellular polymeric substances attached to a surface, this bacterial aggregation being a phenomenon by which microorganisms interact with each other forming a steady, multi-cellular cluster (Abee, Kovacs, Kuipers, & van der Veen, 2011). Biofilm-associated cells can be differentiated from their planktonic counterparts by generation of extracellular polymeric substance matrix, reduced growth rates, and the up- and down-regulation of specific genes (Davey & O'Toole, 2000; Flemming & Wingender, 2010). Biofilm formation is determined not only by the nature of the attachment surface, but also by the characteristics of the bacterial cell and environmental factors. Cell surface hydrophobicity, the presence or absence of some features of the bacterial cell surface (flagella and fimbriae) and the extracellular polymeric substance matrix regulate cell-to-cell and cell-to-surface attachment providing an optimal environment for the exchange of genetic material between cells which may also communicate via quorum sensing (Abee et al., 2011; Lebeer, Verhoeven, Perea Velez, Vanderleyden, & De Keersmaecker, 2007; Van Houdt & Michiels, 2010). In addition, factors that influence microbial adhesion to inert surfaces include physicochemical properties of the surface (roughness, hydrophobicity, and polarity), the presence of organic material and environmental pH and temperature (Van Houdt & Michiels, 2010).

*Listeria monocytogenes* is an opportunistic foodborne pathogen that causes serious illness with a high mortality rate and is frequently isolated from food and food-processing environments (ILSI, 2005; Newell et al., 2010). *L. monocytogenes* is capable of adhering and forming biofilms on food-contact surfaces such as polystyrene, glass and stainless steel and persisting for long periods (Renier, Hebraud, & Desvaux, 2011). The major property of *L. monocytogenes* biofilms, particularly at slaughter and meat processing plants, and on equipment surfaces, is its persistent resistance to desiccation, UV and light, and treatments



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with antimicrobial and sanitizing agents (Sofos & Geornaras, 2010). Biofilm prevention and control is therefore a priority in the food industry; current trends for naturally controlling the shelf life and safety of foods include the use of bacteriocinogenic lactic acid bacteria (LAB) as bioprotective cultures.

In meat and meat products, LAB and their bacteriocins have been well documented for their antimicrobial activity against *L. monocytogenes* (Castellano, Belfiore, Fadda, & Vignolo, 2008; Vignolo, Saavedra, Sesma, & Raya, 2012). LAB were reported to be good candidates to settle protective positive biofilms on food industry surfaces playing a key role in controlling colonization by *L. monocytogenes*. Pathogen inhibition is expected as a result of the modification of the physicochemical properties of the solid surfaces, the competition for nutrients and/or the production of antimicrobial compounds. The settlement of a bacteriocinogenic LAB on surfaces compared to simply conditioning the surface with a bacteriocin has the advantage of limiting nutrient supply by competitive inhibition in addition to bacteriocin production (García-Almendárez, Cann, Martin, Guerrero-Legarreta, & Regalado, 2008; Habimana, Guillier, Kulakauskas, & Briandet, 2011). The ability of *L. monocytogenes* to colonize different surfaces at the low temperatures of food processing and storing, together with the fact that *L. monocytogenes* survives and grows in biofilms at 2 to 4 °C, increases the chance of cross-contamination (Carpentier & Cerf, 2011). Since refrigeration is one of the most common ways to prolong the shelf life of foods, as well as to increase the resistance of bacterial biofilms to antimicrobial treatments, the development of innovative strategies to control this pathogen in food and food processing environments constitutes a major challenge.

Different LAB species have been isolated and identified from meat and meat products (Castro, Palavecino, Herman, Garro, & Campos, 2011; Fontana, Cocconcelli, & Vignolo, 2005, 2006; Fontana, Vignolo, & Cocconcelli, 2005). Biofilm formation by bacteriocin-producing strains may facilitate and promote the colonization of inert materials regularly used in meat processing facilities, thus inhibiting the settlement of pathogens. However, there is no information about the ability of bacteriocinogenic meat borne LAB strains to form biofilms. For this reason an investigation on the adhesive properties under different in vitro conditions and on abiotic surfaces was performed.

## 2. Materials and methods

### 2.1. Microorganisms and culture conditions

Twenty LAB strains isolated from meat and fermented sausages were used (Table 1). Strains were cultivated in MRS broth (10 g/l meat peptone, 10 g/l meat extract, 5 g/l yeast extract, 20 g/l glucose, 1 g/l Tween 80, 2 g/l dibasic ammonium citrate, 5 g/l sodium acetate, 0.2 g/l $MgSO_4 \cdot 7H_2O$, 0.05 g/l $MnSO_4 \cdot 4H_2O$ and 2 g/l $K_2HPO_4$), incubated at 30 °C for 16–18 h and stored at −20 °C in milk yeast extract (10% w/v skim milk, 0.5% w/v yeast extract and 1% v/v glycerol). Additionally, three other media were used for microtiter assays: (i) MRS without Tween 80 (−tMRS), (ii) MRS without Tween 80/glucose/$MnSO_4$ (-tgmMRS) and (iii) modified Luria Bertani (mLB) (10 g/l NaCl, 5 g/l yeast extract and 10 g/l meat peptone). For antagonism activity *Listeria innocua* 7 (kindly provided by the Unité de Recherches Laitieres et Genetique Appliqué, INRA, France) was used and grown in Brain Heart Infusion (BHI) broth at 30 °C. For BioTimer Assay, BioTimer Phenol Red (BT-PR) medium was prepared using MRS as basal media with added phenol red (25 mg/l), glucose (10 g/l) and adjusted to pH 7.2 ± 0.1. The colony forming units (CFU/ml) in BioTimer Assay were determined by the plate count agar method in MRS.

### 2.2. Anti-Listeria activity of LAB strains

The spot-on-lawn assay was used for antimicrobial activity determination of LAB strains against *L. innocua* 7. Overnight cultures of LAB were centrifuged (7000 *g* for 15 min), and then the thermally treated (80 °C, 5 min) supernatant was adjusted to pH 6.5 by adding 10 N NaOH. Supernatants (5 μl) were spotted onto 7 ml of BHI agar plates (0.7% w/v) previously inoculated with 50 μl of *L. innocua* 7. The plates were then incubated at 30 °C for 48 h and the presence of a distinct inhibition zone around the spots was considered as a positive antagonistic effect.

### 2.3. In vitro biofilm assay

Biofilm development by bacteriocinogenic LAB strains was evaluated as described by Lebeer et al. (2007) with minor modifications. Briefly, 200 μl of each tested medium was added to each well of 96 well polystyrene microplates. Overnight LAB cultures from MRS broth were used as inoculum (5% v/v) and incubated without shaking at 30 °C (for 24, 48 and 72 h), and at 10 °C (for 6, 10 and 15 days). To quantify biofilm formation, wells were washed with phosphate-buffered saline (PBS) and the remaining attached bacteria were stained for 30 min with 200 μl 0.1% (w/v) crystal violet in an isopropanol–methanol–PBS solution (1:1:18, v/v/v). Excess stain was rinsed twice with 200 μl distilled water per well. After the wells were air dried, the dye bound to the adherent cells was extracted with 200 μl 30% (v/v) glacial acetic acid. The optical density (OD) of 135 μl of each well was measured at 570 nm using a VersaMax microplate reader (Molecular Devices, Sunnyvale,

**Table 1**
LAB strains used in this study and anti-*Listeria* activity.

| LAB | Strain | Source | Anti-*Listeria* activity | References |
|---|---|---|---|---|
| *Lactobacillus curvatus* | CRL705 | Fermented sausages | + | Vignolo et al. (1993) |
| | CRL1863 | | + | Castro et al. (2011) |
| | CRL1532 | Vacuum-packed beef | + | Fontana et al. (2006) |
| | CRL1533 | | + | |
| | CRL1534 | | + | |
| | CRL1536 | | + | |
| | CRL1537 | | − | |
| | CRL1538 | | + | |
| | CRL1539 | | + | |
| *Lactobacillus sakei* | CRL1463 | Fermented sausages | − | Fontana, Cocconcelli, et al. (2005) |
| | CRL1464 | | − | |
| | CRL1465 | | − | |
| | CRL1466 | | − | |
| | CRL1467 | | − | |
| | CRL1468 | | − | |
| | CRL1469 | | − | |
| | CRL1862 | | + | Castro et al. (2011) |
| | CRL1613 | Vacuum-packed beef | + | Fontana et al. (2006) |
| *Lactobacillus plantarum* | CRL1481 | Fermented sausages | − | Fontana, Cocconcelli, et al. (2005) |
| *Pediococcus acidilactici* | CRL1638 | Fermented sausages | − | Fontana, Vignolo, et al. (2005) |

CA, USA). Sterile medium was included as negative control to ensure that the influence on biofilm formation was not attributed to a nonspecific binding effect to crystal violet. As a selection criterion for biofilm-forming lactobacilli, a cut-off OD for the microtiter-plate test as three standard deviations above the mean OD of the negative control was defined (Stepanović, Vukovic, Dakic, Savic, & Svabic-Vlahovic, 2000). $OD_{570}$ values of different conditions and strains tested 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.

### 2.4. Bacterial cell surface hydrophobic/hydrophilic and Lewis acid–base characteristics

Microbial adhesion to solvent (MATS) was performed according to Bellon-Fontaine, Rault, and van Oss (1996). Three different solvents were used: the nonpolar solvent hexadecane for determining cell surface hydrophobicity/hydrophilicity, and chloroform and ethyl acetate for the acidic–basic character determination. Briefly, LAB were grown for 16–18 h in MRS medium, harvested by centrifugation (7000 *g* for 10 min), washed twice with 0.85% NaCl and resuspended in the same solution to an $OD_{600}$ of 0.3–0.7 ($A_0$). Bacterial suspensions (3 ml) were mixed for 60 s using a vortex (at maximum intensity) with 0.5 ml of each solvent, separately. The mixture was allowed to stand for 15 min, to ensure complete separation of the two phases and the $OD_{600}$ ($A_1$) was measured. The percentage of microbial adhesion to solvent was calculated as $(1 - A_1 / A_0) \times 100$. Each measurement was performed in triplicate and the experiment was repeated twice with independent bacterial cultures.

### 2.5. Autoaggregation assay

Each LAB strain was grown for 16 h at 30 °C in 3 ml MRS and −tMRS and allowed to settle at 10 °C for 24 h. A 0.2 ml aliquot of the upper portion of the suspension was carefully transferred to a microtiter plate, and $OD_{540}$ was measured ($OD_{final}$). A control inoculated tube was agitated for 30 s and $OD_{540}$ was determined ($OD_{initial}$). Autoaggregation percentage was calculated as $[1 - (OD_{final} / OD_{initial})] \times 100$ (Sorroche, Rinaudi, Zorreguieta, & Giordano, 2010).

### 2.6. Biofilm formation on stainless steel and polytetrafluoroethylene chips

#### 2.6.1. Surfaces and cleaning treatment

Surfaces used for biofilm experiments were stainless steel (SS) type AISI 304 (mechanically polished, no. 4 grade; 1.0 cm × 1.0 cm × 1.0 mm thickness), and polytetrafluoroethylene (PTFE), commercially known as Teflon™ (1.0 cm × 1.0 cm × 1.5 mm thickness). Before using, chips were soaked for a minimum of 30 min with acetone, rinsed with distilled water and then soaked again in 1 N NaOH for 1 h. A final rinse with distilled water was carried out and the SS and PTFE chips were air dried.

#### 2.6.2. Development of biofilm on SS and PTFE

After the cleaning treatment SS and PTFE chips were placed in Petri dishes containing 19 ml of sterile MRS and −tMRS media and SS and PTFE chips were inoculated (5% v/v) with overnight biofilm-producing LAB strains and incubated statically at 10 °C for 6 days. Growth media were then removed from the Petri dishes and chips rinsed once with PBS pH 7.2.

#### 2.6.3. BioTimer Assay

Lactobacilli cell count on SS and PTFE chips at 10 °C in MRS and −tMRS was carried out using a modified BioTimer Assay (BTA) as previously described (Pantanella et al., 2008). BTA is an indirect method of quantification to determine the number of bacteria in a biofilm based on the time of turning the indicator phenol red, induced by microbial metabolism. To draw the correlation line specific for each *Lactobacillus* strain, 0.2 ml of MRS overnight broth cultures was mixed with 1.8 ml of BT-PR medium. Two-fold serial dilutions using 1 ml of BT-PR medium were incubated statically in 24-well plates at 30 °C; simultaneously colony forming units (CFU/ml) were determined. The color of the inoculated BT-PR medium was checked at regular intervals. For each two-fold dilution, the time required for a color switch of BT-PR medium was recorded and plotted versus the log of CFU/ml. To quantify the number of cells on SS and PTFE, after biofilm development at 10 °C during 4, 6 and 10 days (samples from 0 to 4 days were avoided due to low lactobacilli growth rate at 10 °C), chips were immersed in BT-PR, incubated at 30 °C and the time of the color change was checked. The time required for color switch was used to determine the number of bacteria in the biofilm on the chips using the correlation line. As the correlation line links the time for color switch of BT-PR medium and the log CFU of planktonic bacteria, the number of cells in the biofilm was expressed as planktonic-equivalent log CFU per chip (PE-log CFU/cm$^2$).

#### 2.6.4. Epifluorescence microscopy

Biofilms developed on SS and PTFE chips were placed in 20 ml of 0.001% (w/v) acridine orange solution to stain attached cells. The chips were then rinsed with distilled water and air dried. Biofilm formation was visualized by a conventional fluorescence microscope (Carl Zeiss Axio Scope A1, Gottingen, Germany) fitted with an appropriated filter for acridine orange. In addition, for the visualization of extracellular polymeric substances, biofilms on SS and PTFE chips were covered with 20 μg/ml FITC labeled concanavalin A (Sigma, Aldrich, MO, USA) solution and incubated in the dark for 1 h. The staining solution was removed by washing the chips twice with lectin buffer (6.057 g/l Tris, 8.7 g/l NaCl, 0.203 g/l $MgCl_2$, 0.111 g/l $CaCl_2$, pH 7.6). Then, cells were counterstained with 2 ng/μl DAPI (Sigma, Aldrich, MO, USA) solution for 15 min in the dark. Samples were observed at 100× magnification with a conventional fluorescence microscope (Carl Zeiss Axio Scope A1, Gottingen, Germany) fitted with appropriated filters for FITC and DAPI.

#### 2.6.5. Scanning electron microscopy (SEM)

Adherent cells in a biofilm were fixed with an 8% (v/v) paraformaldehyde, phosphate buffer and 16% (v/v) glutaraldehyde solution (Karnovsky, 1965) at 4 °C for 16 h. Chips with adhered cells were washed (10 min, three times) with phosphate buffer 0.1 M pH 7.4, post-fixed overnight with 2% (v/v) osmium tetroxide:phosphate buffer (1:1) and washed with 100% ethanol (10 min, twice) after the osmium solution was discarded. Cells were then dehydrated using a graded ethanol series (50, 70, 80, 90, and 100% three times for 10 min each) and subjected to 100% acetone dehydration for 1 h. Chips were sputter-coated with gold and images were taken with a JEOL JSM-35CF (Tokyo, Japan) scanning electron microscope.

## 3. Result and discussion

### 3.1. Anti-Listeria activity of LAB strains

A total of twenty LAB strains, among which nineteen *Lactobacillus* and one *Pediococcus* previously isolated from vacuum-packaged fresh beef and fermented sausages were examined for bacteriocin production (Table 1). Ten out of twenty strains exhibited a high inhibitory effect against *L. innocua* 7, these involved eight *Lactobacillus curvatus* (CRL705, CRL1863, CRL1532, CRL1533, CRL1534, CRL1536, CRL1538 and CRL1539) and two *Lactobacillus sakei* (CRL1613 and CRL1862) strains. These closely related *Lactobacillus* species have been reported to be the most representative bacteria in chilled and packaged raw meat as well as in fermented meat ecosystems (Vignolo, Fontana, & Cocconcelli, 2010). *L. sakei* and *L. curvatus* are considered the representative species from these meat products and are known to produce

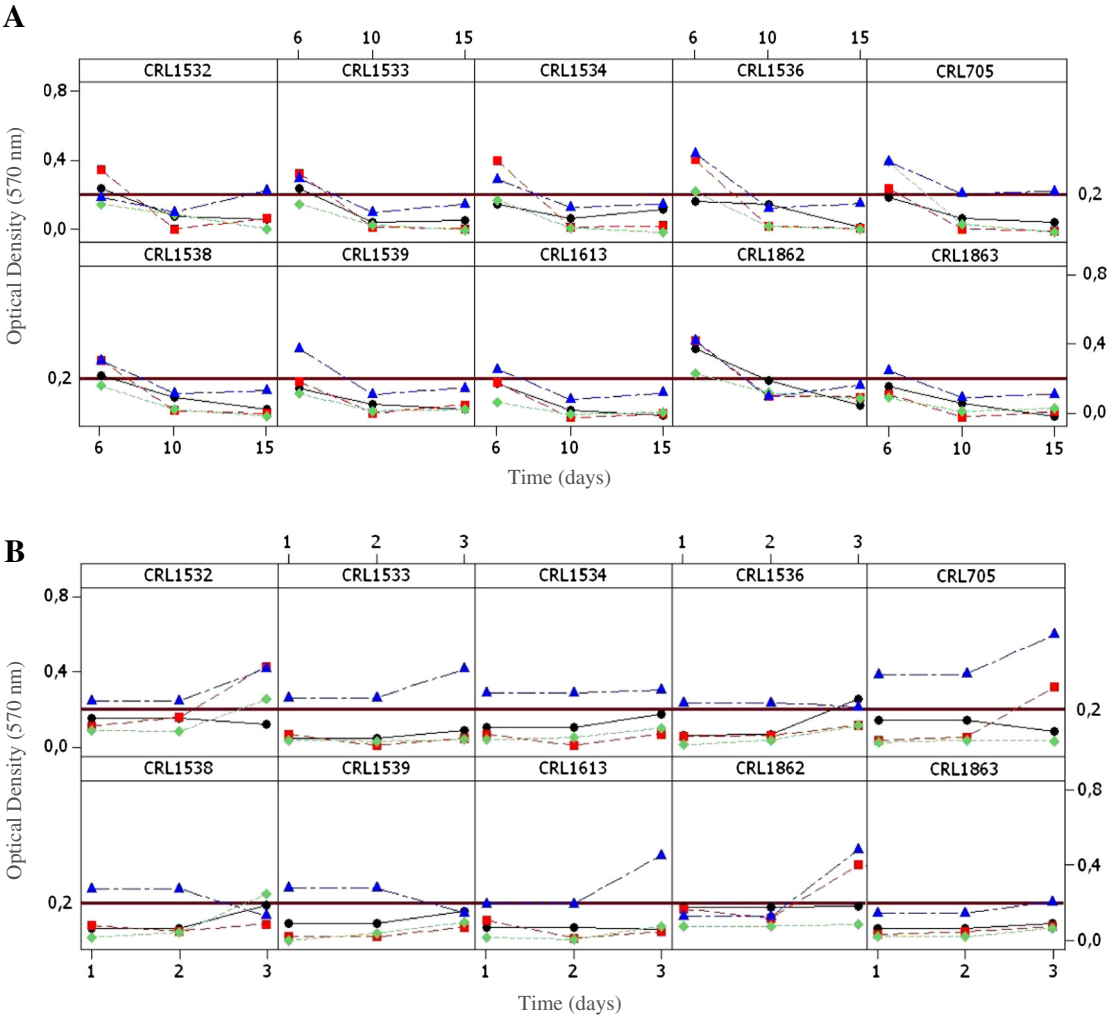


**Fig. 1.** Scatterplot analysis for biofilm formation by ten meat borne *Lactobacillus* strains evaluated at 10 °C (A) and 30 °C (B) during 15 and 3 days, respectively and incubated in MRS (-▲-); −tMRS (-■-); −gtmMRS (-●-) and mLB (-◆-). Cut off line (—).

bacteriocins as an effective and widespread tool for competition in these particular niches. Indeed, bacteriocins produced by LAB isolated from meat and meat products were characterized as belonging to non-lanthionine-containing (class II) peptides. In particular, those active against *Listeria* grouped in the class IIa were reported to be produced by *L. curvatus* and *L. sakei* isolated from fermented sausages (Urso, Rantsiou, Cantoni, Comi, & Cocolin, 2006; Vignolo, Suriani, de Ruiz, Holgado, & Oliver, 1993; Vignolo et al., 2012) and from chilled vacuum-packed raw meat (Jones, Wiklund, Zagorec, & Tagg, 2010). Moreover, comparative genome analysis of *L. curvatus* CRL705 with the closely related *L. sakei* 23K isolated from fermented dry sausages revealed a high similarity between both strains. Genes encoding the production of five different bacteriocins among the protein-encoding genes unique for *L. curvatus* CRL705 were found, highlighting its potential as a biopreservative culture for fresh and processed meat (Chaillou et al., 2005; Hebert et al., 2012).

### 3.2. Screening of lactobacilli for biofilm formation

Selected anti-*Listeria L. curvatus* and *L. sakei* strains were evaluated for biofilm formation in different culture media and temperature–time conditions (10 °C for 6, 10 and 15 days, and 30 °C for 1, 2, and 3 days). Although 30 °C is the optimal growth temperature for the assayed LAB strains, in meat production and storing environments, temperatures below 10 °C are relevant. Results from biofilm formation by the adhesion ability of *Lactobacillus* to polystyrene microtiter plates at different temperatures, incubation times and culture media are shown in Fig. 1A and B. Under the assayed conditions, two different biofilm formation patterns were obtained for lactobacilli strains. Cell adhesion was maximal at 6 days of incubation at 10 °C for all assayed strains (Fig. 1A), while at 30 °C, higher levels of biofilm were formed after 3 days of incubation (Fig. 1B). At maximal adhesion times, scatterplots showed that 9 and 6 lactobacilli strains grown in MRS exhibited $OD_{570\ nm}$ values above

**Table 2**
Percentages of autoaggregation and hydrophobicity of selected *Lactobacillus* strains.

| | Hydrophobicity | | | Autoaggregation | |
|---|---|---|---|---|---|
| | Hexadecane | Chloroform | Ethyl acetate | MRS | −tMRS |
| *L. curvatus* CRL705 | 4.8 ± 2.6 | −8.8 ± 3.9 | 7.5 ± 5.9 | 35.2 ± 1.1 | 63.0 ± 3.2 |
| *L. curvatus* CRL1532 | 3.0 ± 0.2 | −10.2 ± 4.4 | 6.1 ± 0.3 | 14.3 ± 2.1 | 77.8 ± 0.3 |
| *L. sakei* CRL1862 | 0.0 ± 0.0 | −7.0 ± 5.8 | 10.5 ± 3.6 | 49.3 ± 1.4 | 84.4 ± 2.0 |

the cut-off line at 10 and 30 °C, respectively, while in −tMRS medium 6 (10 °C) and 3 (30 °C) bacteriocinogenic strains were able to form biofilms. These results indicate that temperature influenced biofilm formation since more strains were able to form biofilms at 10 °C than at 30 °C. Similar results were reported for *Lactobacillus rhamnosus* strains whose ability to form biofilms increased at lower growth temperature (Emanuel, Adrian, & Diana, 2010). For microorganisms other than LAB, high biofilm formation at suboptimal growth temperature was reported for *L. monocytogenes* and *Staphylococcus aureus* (Chavant, Martinie, Meylheuc, Bellon-Fontaine, & Hebraud, 2002; Rode, Langsrud, Holck, & Moretro, 2007).

The culture media used to investigate biofilm formation led to different levels of adhesion by the tested bacteriocinogenic lactobacilli. From the scatterplots and cut-off lines (Fig. 1A, B) biofilm presence was favored in MRS medium at days 6 and 3 when incubated at 10 and 30 °C, respectively. However, *L. curvatus* CRL1532 and CRL705 and *L. sakei* CRL1862 exhibited an ability to form biofilms not only in MRS but also in −tMRS medium at both assayed temperatures. On the contrary, mLB and -tgmMRS (lacking Tween 80/glucose/$MnSO_4$) media were the least effective for biofilm formation, suggesting that this less-rich medium does not promote lactobacilli adhesion to microtiter plates. Among the individual components evaluated, Tween 80 (a non-ionic surfactant and fatty acid source) did not significantly affect biofilm formation by the assayed meat borne lactobacilli strains in contrast to the inhibitory effect reported for *L. rhamnosus* GG and vaginal lactobacilli (Lebeer et al., 2007; Leccese Terraf, Juarez Tomas, Nader-Macias, & Silva, 2012). The presence of Tween 80 as an emulsifier and dispersing agent to a variety of surfaces was found to inhibit biofilm formation by some Gram-positive and Gram-negative pathogens and clinical isolates (Toutain-Kidd, Kadivar, Bramante, Bobin, & Zegans, 2009). On the other hand, no biofilm production was evident in the present study when glucose (main carbon source for LAB) and $Mn^{+2}$ (growth stimulator) were omitted from MRS, this being in agreement with the biofilm-repressing effect of glucose and divalent cations reported by Lebeer et al. (2007). The nutrient content of the culture medium significantly influences bacterial surface properties and quantity of biofilm produced. The lack of biofilm production by meat borne lactobacilli strains in mLB medium (containing NaCl/peptone/yeast extract) in which the carbon source was omitted agrees with results found for *Lactobacillus acidophilus* (Schar-Zammaretti, Dillmann, D'Amico, Affolter, & Ubbink, 2005) and different *L. rhamnosus* and *Lactobacillus casei* strains (Lebeer et al., 2007). However, when biofilm formation in plastic microtiter-plates was evaluated for *Salmonella* spp. and *L. monocytogenes* the composition of the medium did not have the same influence; a high quantity of biofilm was produced in nutrient-poor and in nutrient-rich media, respectively (Stepanović, Cirkovic, Ranin, & Svabic-Vlahovic, 2004). Therefore, for biofilm formation a high dependence on the strain, chemical composition of the culture medium and physicochemical factors has to be recognized.

### 3.3. Evaluation of cell surface properties

*L. curvatus* CRL1532 and CRL705 and *L. sakei* CRL1862 were selected based on their ability to form biofilms at 10 and 30 °C under different culture conditions and their surface properties were assessed. Using MATS partitioning method, lactobacilli strains were tested for their affinity to chloroform (acidic solvent-electron acceptor), ethyl acetate (basic solvent-electron donor) and hexadecane (nonpolar solvent). Anti-*Listeria* lactobacilli strains showed low adhesion to the assessed hydrocarbons (Table 2). However, slight affinity to hexadecane was exhibited by *L. curvatus* CRL705 (4.8 ± 2.6%) and CRL1532 (3.0 ± 0.2%) while no affinity to this solvent was found for *L. sakei* CRL1862 indicating limited adhesion of lactobacilli to this nonpolar solvent. *L. sakei* CRL1862 showed the highest affinity value (10.5 ± 3.6%) to ethyl acetate, followed by *L. curvatus* CRL705 (7.5 ± 5.9%) and *L. curvatus* CRL1532 (6.1 ± 0.3%) while no adhesion to chloroform was exhibited by the three strains. Due to the low adhesion percentages to hexadecane, a rather hydrophilic surface for lactobacilli strains could be inferred. The low affinity for hexadecane obtained in this study agrees with results for dairy strains (*L. casei*, *L. rhamnosus* and *Lactobacillus paracasei*) in which values ranged from 2.7 to 26.5% (Pelletier et al., 1997) and for sugar refinery plant isolates (*Leuconostoc mesenteroides* and *Streptococcus thermophilus*) with adhesion values lower than 20% (Bellon-Fontaine et al., 1996). As measured by MATS, the surface characteristics of meat borne *L. sakei* CRL1862 and *L. curvatus* CRL705 may be considered of low hydrophobicity according to values reported for oral lactobacilli (Samot, Lebreton, & Badet, 2011). This relatively hydrophilic global nature of LAB, regardless of the genera and species has been found in other studies (Ly, Vo, Le, Belin, & Wache, 2006).

Bacterial adhesion to chloroform and ethyl acetate was performed to assess the Lewis acid–base cell surface characteristics. The higher adhesion to basic solvent (ethyl acetate) than to acidic solvent (chloroform) displayed by *L. sakei* CRL1862 and *L. curvatus* CRL705 may indicate a slightly acidic cell surface character (electron acceptor) in contrast to those found for dairy LAB (Giaouris, Chapot-Chartier, & Briandet, 2009; Pelletier et al., 1997) and *Staphylococcus* strains (Planchon et al., 2006). Due to the fact that lactobacilli strains are strong lactic acid producers, more electron acceptor groups would be present on their cell wall so, some affinity with the electron donor solvent (ethyl acetate) should be displayed. The presence of bound acidic compounds on lactobacilli cell walls may explain the acidic character of CRL1862 and CRL705 strains, this phenomenon

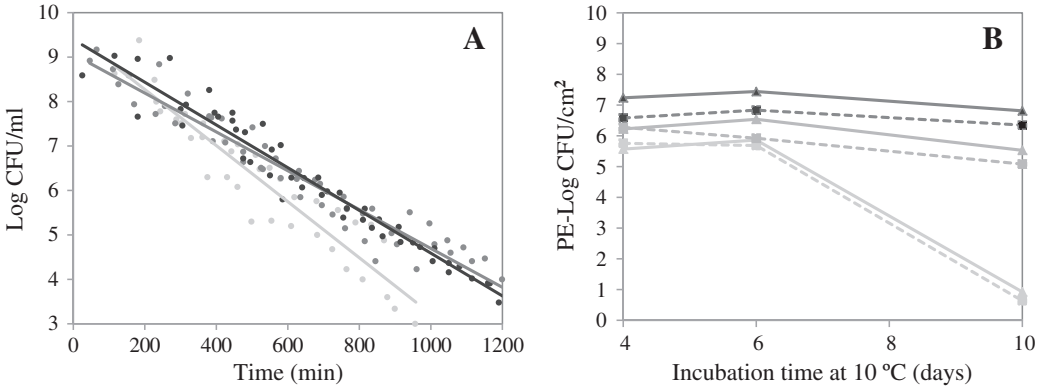


**Fig. 2.** BioTimer Assay. (A) Correlation lines linking the time for color switch of BT-PR medium and the number of planktonic cells for *L. curvatus* CRL705 (light gray), *L. curvatus* CRL1532 (dark gray) and *L. sakei* CRL1862 (black); (B) lactobacilli strain adhesion to SS (continuous line) and PTFE (dashed line) during incubation at 10 °C for 10 days. PE-log CFU/$cm^2$: planktonic-equivalent log CFU per chip.

being also described for *L. monocytogenes* (Briandet, Meylheuc, Maher, & Bellon-Fontaine, 1999). As a whole, high diversity of surface physicochemical properties among LAB not connected to the genera/species and the origin of the isolates was reported, including strains with a wide range of affinity percentages to different solvents (Giaouris et al., 2009; Ly et al., 2006).

The cell-to-cell aggregation ability of the three selected *Lactobacillus* strains on the basis of their sedimentation characteristics was evaluated after 24 h at 10 °C. The three strains exhibited autoaggregation values between 14 and 49% in MRS and 63 and 84% in −tMRS medium, *L. sakei* CRL1862 showing the strongest autoaggregative profile. A higher autoaggregation level was found when Tween 80 was omitted in the MRS medium, which is in line with the dispersing effect of this surfactant. However, different levels of autoaggregation have been reported for LAB, values ranging from around 30% to more than 90% for dairy strains (Nikolic, Jovcic, Kojic, & Topisirovic, 2010) and between 72 and 80% for human isolates such as *Bifidobacterium* and *L. acidophilus* (Del Re, Sgorbati, Miglioli, & Palenzona, 2000; Kos et al., 2003). Based on the autoaggregation percentages found, *L. sakei* CRL1862 would be defined as presenting a strong autoaggregating phenotype while an intermediate aggregative characteristic for *L. curvatus* CRL705 and CRL1532 is suggested.

Nevertheless, in contrast to the positive correlation of autoaggregative dairy *Lactobacillus* and *Leuconostoc* isolates with high adhesion to nonpolar hydrocarbons reported by Nikolic et al. (2010), the highest autoaggregative meat borne strain *L. sakei* CRL1862 showed weak affinity to ethyl acetate. Even though a general correlation between bacterial overall surface characteristics (adhesion to hydrocarbons) and binding capabilities (autoaggregation) has been stated, each strain should be examined on a case-by-case basis. Based on the cell surface properties of bacteriocinogenic *Lactobacillus* strains, the ability to autoaggregate together with the low hydrophilic character of the cell surface as measured by MATS could be used for preliminary screening to identify potentially biofilm forming bacteria on surfaces of industrial interest.

### 3.4. Lactobacilli biofilm on SS and PTFE chips

The ability of meat borne lactobacilli to form biofilms on SS and PTFE chips was investigated. The BioTimer Assay (BTA) was used to detect the bacterial number forming biofilm. BTA is a reliable, sensitive, rapid and easy-to-perform method used as a valuable tool to count adherent bacteria on different surfaces, in contaminated foods, and to analyze antibiotic susceptibility without sample manipulation (Pantanella et al., 2008). A correlation line linking the time for the color switch of BT-PR medium and the number of planktonic *Lactobacillus* cells at 30 °C was drawn. The data showed a linear correlation between time for color switch and log of CFU of initial bacterial concentration for each strain. The equations and the linear correlation coefficients describing the correlation lines were calculated for each *Lactobacillus* strain on the whole data set (Fig. 2A). Counts of lactobacilli numbers in the biofilm and expressed as PE-log CFU/cm$^2$ are shown in Fig. 2B. For each *Lactobacillus* strain, similar patterns for cell numbers in biofilm adherent on colonized SS and PTFE chips were observed during incubation in MRS at 10 °C from 4 to 10 days. Cell counts were slightly higher on SS compared to PTFE for the three strains, showing a preferential adhesion on SS (Fig. 2B). Maximal cell numbers in the biofilm were reached after 6 days of incubation at 10 °C, *L. sakei* CRL1862 exhibiting the highest values (7.44 PE-log CFU/cm$^2$) on SS and (6.84 PE-log CFU/cm$^2$) on PTFE with a decreasing tendency towards day 10. A slightly lower cell count profile was observed for *L. curvatus* CRL1532 while a dramatic decline after 6 days was detected for *L. curvatus* CRL705 counts in the biofilm. When lactobacilli numbers in the biofilm on SS and PTFE were evaluated in −tMRS medium, one log cycle lower planktonic-equivalent CFU than those counted in MRS medium was found (data not shown).

Although SS is of high cost and susceptible to corrosion it is still the most frequently used material for food processing equipment. However, more recently plastic polymers have become popular and are used in the food industry for the construction of conveyor belts, tanks, pipework and cutting boards. Among plastics, PTFE commercially known as Teflon™ is an increasingly used material in food-processing coatings for its unique properties (Pompermayer & Gaylarde, 2000). In this study, it was shown that regardless of *Lactobacillus* species, maximal cell counts in the biofilm were recorded on SS surfaces during incubation in MRS medium at 10 °C. It is well known that microbial adherence to inert surfaces depends on bacterial characteristics and on the material; the greater biofilm formation on SS in this study agrees with the relative hydrophilic character of lactobacilli surfaces. Therefore, the higher number of *L. sakei* CRL1862 cells in the biofilm on SS correlated with cell affinity to ethyl acetate, this being consistent with the physicochemical characteristics of ethyl acetate and SS which have strong electron donor properties and weak electron acceptor character. Although the hydrophilic nature of SS has long been recognized (Chavant et al., 2002; Flint, Brooks, & Bremer, 2000; Planchon et al., 2006) it was also described as hydrophobic (Brugnoni, Lozano, & Cubitto, 2007), a fact that was associated with the chemical composition and surface finishing of SS as well as the methods used to measure hydrophobicity (Flint et al., 2000). In agreement with the results from this study, the rate of adhesion of thermo-resistant dairy streptococci and *L. monocytogenes* Scott A was enhanced in the presence of a hydrophilic substrate such as SS (Briandet et al., 1999; Flint et al., 2000). On the other hand, although surface colonization was evident at 10 °C, the adherent population of *L. sakei* CRL1862 on PTFE was not as high as on SS. It can be hypothesized that the combination of the relative hydrophilic cell envelope of *Lactobacillus* and the hydrophobic PTFE surface led to significantly lower colonization, as reported for *L. monocytogenes* adhesion on hydrophobic substrates (Chavant et al., 2002). The biofilm formation ability found for *L. sakei* CRL1862 in this study is in contrast to the inability of this species to survive and adhere to abiotic surfaces (Ammor et al., 2005), but in agreement with those

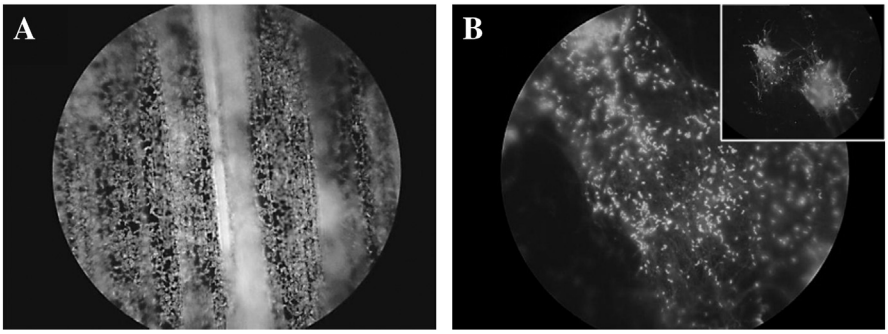


**Fig. 3.** Epifluorescent micrographs of *L. sakei* CRL1862 attached to SS (A) and PTFE (B) chips after incubation at 10 °C for 6 days. Biofilms were stained with acridine orange and visualized using fluorescent microscopy. Magnification 100×.

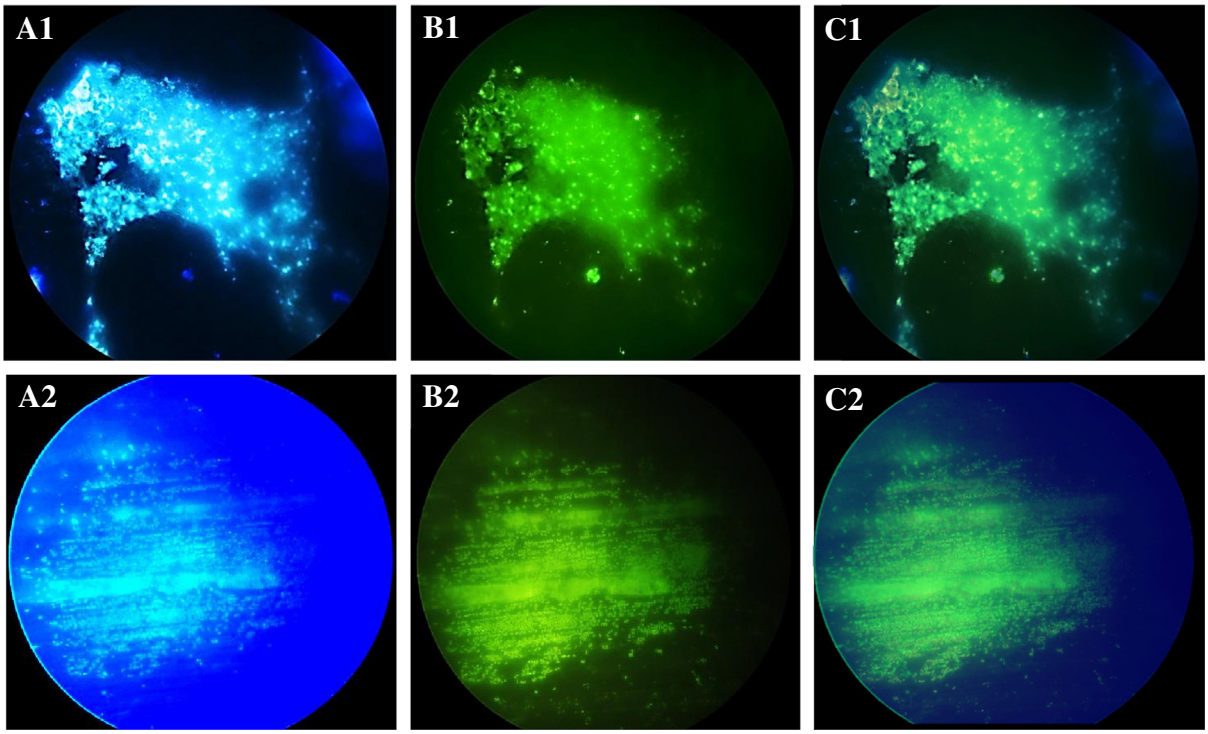


**Fig. 4.** Fluorescence microscopy observations of *L. sakei* CRL1862 biofilm on PTFE (1) and SS (2). (A) Biofilm distribution on the surface (DAPI staining); (B) extracellular polymeric substance presence covering the biofilm in green (ConA-FITC staining) and (C) the two images merged. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

reported for the *L. sakei* 23K genome sequence in which a set of genes potentially responsible for biofilm formation and cellular adherence were present (Chaillou et al., 2005).

### 3.5. *L. sakei CRL1862 biofilm characterization*

Photomicrographs of maximal cell number for *L. sakei* CRL1862 biofilm formation on SS and PTFE taken by epifluorescence microcopy are shown in Fig. 3. Biofilm formation homogeneously almost entirely covered the SS chip surface (Fig. 3A), while cell clusters on PTFE were observed (Fig. 3B). These results are in agreement with the relative hydrophilic surface of *L. sakei* CRL1862 that leads to better colonization on SS than on PTFE. In particular, the photomicrographs of the epifluorescent-stained SS samples often revealed lactobacilli cells aligned with the striations of the visible manufacturing flaws. A similar adhesion pattern for biofilm

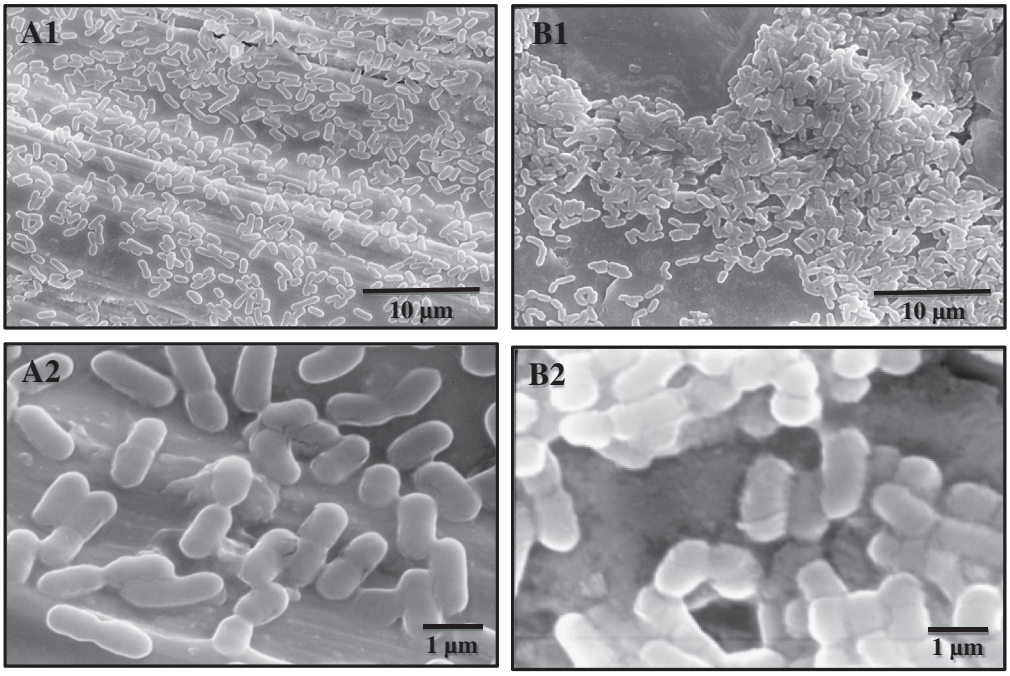


**Fig. 5.** SEM images of biofilm forming *L. sakei* CRL1862 in MRS after incubation at 10 °C for 6 days on SS (A) and PTFE (B) at low (A1, B1) and high (A2, B2) magnifications.

formation on SS (AISI 304) was reported for thermo-resistant streptococci (Flint et al., 2000). In addition, adhesion of *L. sakei* CRL1862 on PTFE showed that aggregated cells were packed together very closely and appeared embedded in a cloud of clearly visible matrix material linked by filamentous ramifications suggesting the presence of DNA/RNA cellular material. Using fluorescent labeled lectin (concanavalin), the presence of carbohydrates in the self-produced matrix of extracellular polymeric substances in the biofilm was visualized (Fig. 4). The diffuse appearance of bacterial cells in the large microcolonies suggests that these bacteria are surrounded by extracellular polymeric substances. Flemming and Wingender (2010) reported that there is no biofilm without an extracellular polymeric substance matrix which is essential to ensure mechanical stability, adhesion to surfaces and to form a cohesive, three-dimensional network that interconnects and transiently immobilize biofilm cells. By SEM, the biofilm formed by *L. sakei* CRL1862 on SS and PTFE after growth in MRS during 6 days at 10 °C (Fig. 5) showed cell adhesion patterns similar to those observed by epifluorescence microscopy, which depended on the abiotic surface characteristics. Noticeably, the extracellular polymeric substance matrix of formed biofilm was different on each surface showing adaptation to the material topography. Indeed, the extracellular polymeric substance matrix covering along the SS scratches resulted in a smoothing of the microstructure in contrast to the amorphous extracellular polymeric substance mass surrounding bacterial clusters formed on PTFE surface (Fig. 5).

To conclude, the screening of anti-*Listeria* meat borne LAB strains for biofilm production allowed the selection of *L. sakei* CRL1862 which was able to grow on both, SS and PTFE, materials frequently used in food processing premises. Studies on the inhibition of *L. monocytogenes* by bacteriocinogenic LAB in biofilms are in progress with a view to develop new strategies for controlling this pathogen.

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