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Lactic acid bacteria biofilms and their ability to mitigate *Escherichia coli* O157:H7 surface colonization

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SIGNIFICANCE AND IMPACT OF THE STUDY:

Nowadays, the use of LAB (Lactic Acid Bacteria) in food processing environments is considered as a biological strategy to control food-borne pathogens. This work provides new insights about the capacity of LAB to form biofilms and to inhibit growth and surface colonization of Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 under usual meat-processing environments. Our findings support the use of biofilm-forming LAB strains as a biological strategy to control EHEC contaminations from food processing surfaces.

ABSTRACT

LAB (Lactic Acid Bacteria) exert antagonistic activities against diverse microorganisms, including pathogens. In this work, we aimed to investigate the ability of LAB strains isolated from food to produce biofilms and to inhibit growth and surface colonization of Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 at 10°C. The ability of 100 isolated LAB to inhibit EHEC O157:H7 NCTC12900 growth was evaluated in agar diffusion assays. Thirty-seven LAB strains showed strong growth inhibitory effect on EHEC. The highest inhibitory activities corresponded to LAB strains belonging to *Lactiplantibacillus plantarum*, *Pediococcus acidilactici* and *Pediococcus pentosaceus* species. Eighteen out of the thirty-seven strains that showed growth inhibitory effects on EHEC also had the ability to form biofilms on polystyrene surfaces at 10°C and 30°C. Pre-established biofilms on polystyrene of four of these LAB strains were able to reduce significantly surface colonization by EHEC at low temperature (10°C). Among these four strains, *Lact. plantarum* CRL 1075 not only inhibited EHEC, but also was able to grow in the presence of the enteric pathogen. Therefore, this strain proved to be a good candidate for further technological studies oriented to its application in food processing environments to mitigate undesirable surface contaminations of *E. coli*.

Keywords: BIOCONTROL, BIOFILM, *ESCHERICHIA COLI* O157:H7, LACTIC ACID BACTERIA, SURFACE COLONIZATION

INTRODUCTION

Biofilms are sessile bacterial communities formed on surfaces encased in an extracellular matrix of polymers that provides adhesiveness, cohesion and protection (Flemming and Wingender 2010; Abee *et al.* 2011). The ability of bacteria to attach to abiotic surfaces and to form biofilms is a major cause of concern for the food industry, particularly in meat production, processing and packaging (Chmielewski and Frank

2003). In fact, biofilm formation improves the capacity of foodborne bacteria to survive stressful conditions found during food processing such as refrigeration, acidity or oxidative and osmotic stress. A large number of EHEC O157:H7 outbreaks have been associated with the consumption of contaminated ground beef (Omer *et al.* 2018). This pathogen has the ability to form biofilms on different materials used in the meat processing industry, such as stainless steel, plastic or glass. (Uhlich *et al.* 2006; Oloketuyi and Khan 2017). Thus, EHEC O157:H7 biofilms established on inadequately cleaned and sanitized meat processing facilities becomes a major source of meat contamination, leading to serious hygienic problems and economic losses (Sharma *et al.* 2005).

Cleaning and disinfection procedures using physical and chemical methods have been extensively used over the years to reduce or eliminate microorganisms present on food contact surfaces. Nevertheless, current sanitation methods have some drawbacks, such as possible toxicity or resistance to sanitization agents developed by the target microorganisms (Langsrud *et al*. 2004; Moen *et al.* 2012).

The growing negative consumer perception against synthetic chemicals, has redirected the research focus towards the development of environmental-friendly disinfection alternatives. Among them we can mention the use of biological strategies, including the use of natural compounds from bacteria or plants with GRAS (Generally Recognized As Safe) status, or even bacteriophages (Donlan *et al.* 2009; Neyret *et al.* 2014).

In this context, numerous studies have showed the ability of lactic acid bacteria (LAB) to exclude unwanted bacteria (Ouali *et al.* 2014; Gómez *et al.* 2016). LAB are considered as GRAS and their use in the food industry constitutes a promising biological strategy against pathogens. LAB can potentially antagonize attachment and growth of pathogens onto abiotic surfaces indirectly, by secreting antimicrobial compounds like heat stable bacteriocins, organic acids and surfactants; or directly, by limiting access to surfaces and nutrients (competitive exclusion) (Pérez-Ibarreche *et al.* 2016; Alvarez-Ordóñez *et al.* 2019). Thus, LAB strains hold promise as biocontrol agents of biofilm-forming pathogens on food industrial environments, without posing any risk to consumers.

In the present study, we investigated the capacity of LAB isolates to form biofilms and to inhibit the growth and surface colonization of EHEC O157:H7 at 10°C, a usual temperature in meat-processing environments. Our findings support the use of biofilm-forming LAB strains as a biological strategy to eliminate EHEC contaminations from food processing surfaces.

RESULTS AND DISCUSSION

Growth inhibitory effect of LAB strains on EHEC O157:H7

To examine the potential growth inhibitory effects of 100 LAB strains on EHEC O157:H7 NCTC12900, we used the agar diffusion assay. Briefly, pure cultures or fractions of them containing either cells or heat-treated/neutralized supernatants and as acidity control, were spotted on MRS (de Man Rogosa and Sharpe) agar. As acidity control, 4% lactic acid was included. Thereafter, a top agar containing EHEC O157:H7 NCTC12900 cells was overlaid.

Notably, while 56 LAB strains exhibited intermediate anti-EHEC activity, 37 strains showed high antagonistic activity on EHEC. Strains having high inhibitory effects belong mainly to *Lact. plantarum* (10), *Ped. acidilactici* (7) and *Ped. pentosaceus* (6) species and were isolated mainly from artisanal sausages and cabbages (Table 1). The inhibitory activities were observed only when EHEC was exposed to culture fractions containing concentrated cell suspensions or whole liquid cultures of LAB strains (Table 1). When EHEC was challenged with heat-treated and/or neutralized supernatants from cultures of the same strains, the inhibitory effects on the pathogen were no observed (data not shown). These results indicate that neither heat-stable bacteriocins nor acids (usually present in supernatants of LAB cultures) are involved in the inhibitory effect on EHEC. This agrees with previous findings by Orihuel et al. (2018) who did not observe any inhibitory activity against EHEC by culture supernatants of different LAB strains. Thus, we can infer that the inhibitory activity of LAB requires the presence of viable cells. This could be associated to a Contact-dependent growth inhibition (CDI) mechanism. Bacteria may deliver toxin molecules into neighbouring bacteria upon direct cell-cell contact, causing growth arrest or cell death (Ruhe *et al.* 2013). A similar inhibition mechanism has been recently reported for the interaction between *Lactococcus piscium* and *Listeria monocytogenes* (Saraoui *et al.* 2018).

Table 1. LAB strains showing high antagonistic activity against EHEC O157:H7 NCTC12900 by well-diffusion assay

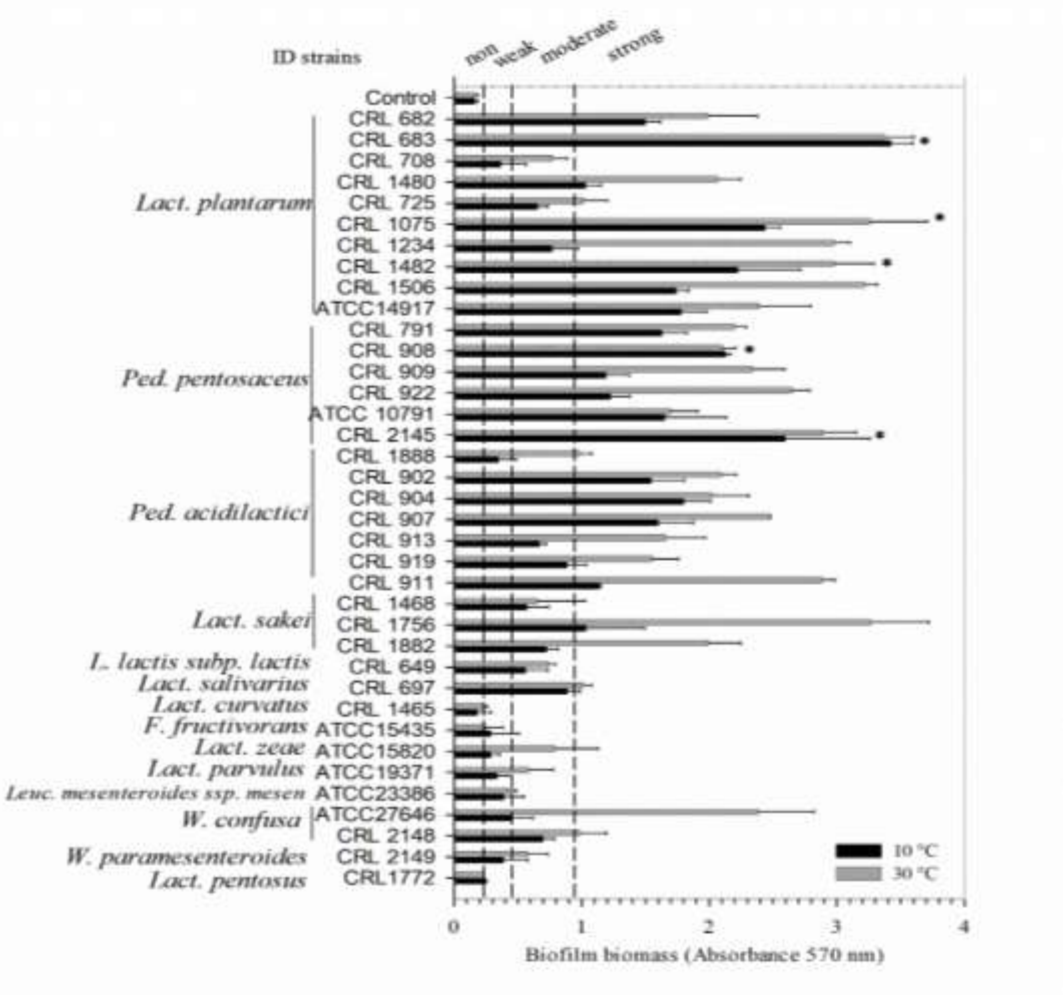
			Zone of inhibition average from triplicate spots[a] (mm)		
LAB species	**Strain ID**	**Origin**	**Spot 1**	**Spot 2**	**Score[b]**
Lactiplantibacillus plantarum	CRL682	Sausages	6.7	4	++/++[c]
	CRL683	Sausages	6	4	++/++
	CRL708	Sausages	4.7	4.1	++/++
	CRL 1480	Sausages	6	4.3	++/++
	CRL 725	Sausages	6	4	++/++
	CRL 1075	Peas	6	4	++/++
	CRL 1234	Cabbage	7	5	++/++
	CRL 1482	Fermented sausages	6	4	++/++
	CRL 1506	Goat milk	6	4	++/++
	ATCC14917	Pickled cabbage	6	5	++/++
Pediococcus pentosaceus	CRL 791	Cabbage	6	5	++/++
	CRL 908	Cabbage	6	4	++/++
	CRL 909	Cabbage	5	5	++/++
	CRL 922	Cabbage	6	4.1	++/++
	ATCC 10791	Pickled cucumber	6.7	5.7	++/++
	CRL 2145	Chickpea sourdough	6	6	++/++
Pediococcus acidilactici	CRL 1888	Sausages	5	5	++/++
	CRL 902	Cabbage	6	4.7	++/++
	CRL 904	Cabbage	6	6	++/++
	CRL 907	Cabbage	6	5	++/++
	CRL913	Sausages	4	4	++/++
	CRL919	Sausages	4	4	++/++
	CRL 911	Argentinean artisanal fermented sausages	5	4	++/++
Lactilactobacillus sakei	CRL 1468	Sausages	5	4	++/++
	CRL 1756	Fresh anchovies	6	6	++/++
	CRL 1882	Sausages	6	5	++/++
Lactococcus lactis sub lactis	CRL 649	Sausages	6	4	++/++
Ligilactobacillus salivarius	CRL697	Sausages	6.7	5.3	++/++
Latilactobacillus curvatus	CRL 1465	Sausages	6	6	++/++
Fructilactobacillus fructivorans	ATCC15435	Contaminated sake	7	6	++/++
Lactobacillus zeae	ATCC15820	Macerated corn	7	5	++/++
Pediococcus parvulus	ATCC19371	Ensilage	6	4	++/++
Leuconostoc mesenteroides subp. mesenteroides	ATCC23386	Sake starter culture	6	6	++/++
Weisella confusa	ATCC27646	Lettuce leaves	7	6	++/++
	CRL 2148	Bean sourdough	6	6	++/++
Weisella paramesenteroides	CRL 2149	Bean sourdough	6	4	++/++
Lactiplantibacillus pentosus	CRL 1772	Olives brine	4	4	++/++

[a]spot designations: 1- Concentrated cell suspension; 2- overnight pure culture of the LAB strain in MRS. [b]Anti-EHEC activity score according to the diameter of the zone of inhibition: – ≤1.0 mm; + =1.1–3.9 mm and ++ ≥4.0 mm. [c] Strains with high anti-EHEC activity ++ / ++ present ≥4 mm inhibition zone in spots 1 and 2.

Biofilm formation by selected LAB strains on abiotic surfaces

As next step in the evaluation of LAB candidates that antagonize EHEC, we examined the ability to form biofilms of the 37 LAB strains with high anti-EHEC activity. All strains were evaluated for biofilm formation on polystyrene surfaces at two temperatures: 10°C, which reflects temperatures in meat processing environments, and 30°C, optimal growth temperature for LAB. Biofilm formation was assessed by Cristal violet (CV) staining and the strains were classified as strong, moderate, weak biofilm producers or non-biofilm producers based on the CV absorbance (A_{570}) values. As shown in Figure 1, eighteen LAB strains showed high capacity to form biofilms at both 10°C and 30°C with A_{570} values between 0.95 and 3.50. Among them, five LAB strains (*Lact. plantarum* CRL 683, *Ped. pentosaceus* CRL 908, *Lact. plantarum* CRL 1075, *Lact. plantarum* CRL 1482 and *Ped. pentosaceus* CRL 2145) resulted strong biofilm-producers at both temperatures (Figure 1). These five LAB strains were then selected for further studies. Although, Emanuel et al. (2010) and Pérez-Ibarreche et al. (2016) have previously reported that LAB ability to form biofilms increased at low temperature, our strains showed greater biofilm-forming capacity at 30ºC. This corroborates that biofilm formation and its optimal conditions are strain-dependent.

Fig. 1 Biofilm-forming capacity of antagonistic LAB strains on polystyrene surfaces at 10ºC for 48 h. Strains are classified as strong, moderate, weak or no biofilm producers. Each bar represents the mean value of at least three independent experiments performed in quadruplicates. Asterisks indicate strains with the highest absorbance values at both temperatures. Error bars represent standard error.



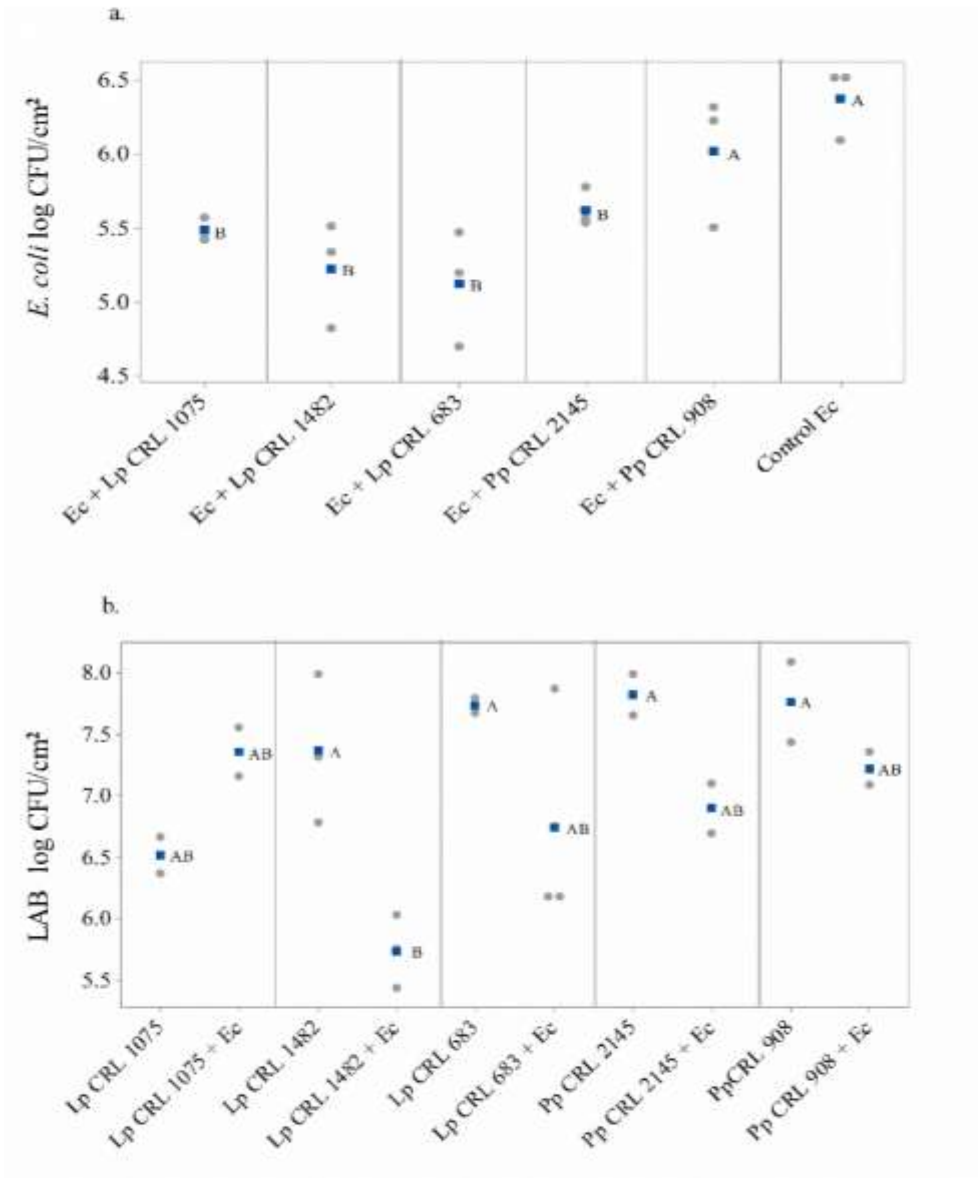
Potential of sessile LAB strains to inhibit the settlement of EHEC biofilm on abiotic surfaces.

Exclusion assays at 10°C

Considering the potential application of LAB strains as biocontrol agents in meat processing environments, we examined whether pre-formed biofilms on polystyrene of the five selected LAB strains were able to prevent the colonization of the surface by EHEC NCTC12900 at low temperature (10°C). As shown in Figure 2a, pre-established biofilms of *Lact. plantarum* CRL 683, *Lact. plantarum* CRL 1075, *Lact. plantarum* CRL1482 and *Ped. pentosaceus* CRL 2145 reduced significantly the colonization of EHEC after 24 h of co-incubation. The remaining number of viable EHEC cells was about the minimal infective dose of this pathogen (10-100 cells/g of food) (Law *et al.* 2000). However, it must be considered that the number of *E. coli* cells on meat-processing surfaces is usually significantly lower than the inoculum used here, in this assay. Thus, it seems logical to speculate that in a real scenario in the food industry, biofilm-forming LAB strains could exclude EHEC contaminations.

Lact. plantarum CRL 1075 deserves special consideration as potential biocontrol strain. This strain reduced the number of viable EHEC and was the only one whose cell population grew in the presence of the pathogen (Figure 2b). Previous studies have also demonstrated the ability of LAB to reduce the colonization of food-borne pathogens, however, these studies were carried out at temperatures higher than 10°C. In fact, Merino et al. (2019), have reported that *Lact. kefiri* 83113 inhibits *Salmonella* 115 biofilm formation at 28°C. Similarly Gómez et al. (2016) have demonstrated the ability of probiotic LAB biofilms to prevent *Listeria monocytogenes, Salmonella* Typhimurium and EHEC O157:H7 biofilm formation through exclusion mechanisms at 30°C. We believe that our studies at 10°C provide new insights into the antagonistic potential of LAB strains in an environment closer to that in the meat-processing industry.

Fig. 2 Exclusion assays at 10°C in a meat-based medium. EHEC NCTC12900 challenged with pre-established biofilms of LAB in a meat-based medium at 10°C for 24 h on polystyrene microplates. a) EHEC cells (Ec) counting (log CFU/cm^2) in absence (control Ec) or presence of *Lact. plantarum* (Lp) CRL 1075, Lp CRL 1482, Lp CRL 683, *Ped. pentosaceus* (Pp) CRL 2145, Pp CRL 908 biofilms. b) LAB cell counting (log CFU/cm^2) after biofilm growth either alone or subsequently challenged with EHEC cells. The squares represent the mean value and the dots represent each independent experiment value. One-way analysis of variance and Dunnet or Tukey test was applied. Different letters indicate statistically significant differences between the groups (p<0.05).



Evaluation of antibiotic susceptibility

Recently, the role of food-associated bacteria acting as reservoir of antibiotic resistance determinants has gained special attention in the food industry (Devirgiliis *et al.* 2011; González-Zorn and Escudero 2012). Thus, the assessment of antimicrobial susceptibility of bacteria intended to be used in food has become a mandatory step in the food safety management. In this regard, the EFSA (European Food Safety Authority) has provided phenotypic methods for determining susceptibility to antimicrobials (EFSA *et al.* 2012). It also recommends defining the genetic basis of the resistance, looking at acquired or transferable determinants. Considering the potential application of LAB as biocontrol agents, we evaluated the antibiotic susceptibility of the five selected strains (*Lact. plantarum* CRL 683, CRL 1075, CRL 1482 and *Ped. pentosaceus* CRL 908, CRL 2145) by determining the Minimum Inhibitory Concentration (MIC) of eight antibiotics of clinical and veterinary importance: ampicillin; chloramphenicol; tetracycline, erythromycin, gentamicin, kanamycin, clindamycin and streptomycin. As shown in Table 2, the five selected LAB strains were susceptible to chloramphenicol, erythromycin, gentamicin, clindamycin and streptomycin. Nevertheless, the five LAB strains presented resistance to ampicillin and tetracycline and the two *Ped. pentosaceus* strains also showed resistance to kanamycin.

Table 2. Minimum inhibitory concentration (MIC) of antibiotics against selected LAB strains

Strains	**MIC[a] (mg l^{-1})**							
	AMP	**CMP**	**TET**	**ERY**	**GEN**	**KAN**	**CLI**	**STR**
Lact. plantarum CRL 683	4 (R)	8 (S)	64 (R)	<0.125 (S)	2 (S)	32 (S)	0.5 (S)	n.r
Lact. plantarum CRL 1075	4 (R)	4 (S)	64 (R)	<0.125 (S)	2 (S)	32 (S)	1 (S)	n.r.
Lact. plantarum CRL 1482	4 (R)	4 (S)	64 (R)	<0.125 (S)	<1(S)	32 (S)	1 (S)	n.r.
Ped. pentosaceus CRL 908	8 (R)	4 (S)	64 (R)	<0.125 (S)	2 (S)	128 (R)	<0.125 (S)	64 (S)
Ped. pentosaceus CRL 2145	8 (R)	4 (S)	64 (R)	<0.125 (S)	4 (S)	128 (R)	<0.125 (S)	64 (S)

[a] MIC of the following antibiotics: AMP, ampicillin; CMP, chloramphenicol; TET, tetracycline; ERY, erythromycin; GEN gentamicin; KAN, kanamycin; CLI, clindamycin and STR, streptomycin. Classification of strains according to the cut-off values of MIC described in the EFSA guidelines [EFSA, 2012]: (R), resistant; (S), Susceptible; n.r., not required

When the presence of transferable antibiotic resistance genes was investigated by PCR, only the *tet(M)* gene encoding ribosomal protection protein for tetracycline resistance was detected in two strains (*Lact. plantarum* CRL 683 and *Ped. pentosaceus* CRL 908) (data not shown). These strains generated a PCR product of the expected size (406-bp) amplified with primers specific to the *tet(M)* gene (nt 1472731-1473136; 100% identity) of *Staphylococcus aureus* (Genbank accession no. AP024511) (Malhotra-Kumar *et al.* 2005). The *tet(M)* gene has been reported to be associated to the Tn*916* transposon (Devirgiliis *et al*.

2013). Although the flanking regions of *tet(M)* have not been characterized in our strains, the presence of a transposon-associated *tet(M)* indicated that the two strains should not be considered for use in the food industry due to their high potential for horizontal tetracycline-resistance gene propagation. Other tetracycline resistant determinants, such as *tet(W), tet(K), tet(L), tet(S)* and *tet(O)*, were not detected in any of the selected strains. Similarly, it was not possible to detect resistance markers in those strains with resistance against ampicillin and kanamycin (data not shown). The level of resistance to these antibiotics exceeded a dilution of the breakpoints established by EFSA. MIC breakpoints may allow detection of some "borderline" resistant strains (Hummel *et al.* 2006; Połka *et al.* 2016). This could be due to the inherent insensitivity of some of the strains to certain antibiotics due to complex intrinsic characteristics such as cell wall/ membrane impermeability or metabolic properties (Hummel *et al.* 2007; Gueimonde *et al.* 2013). This resistance may not be associated with horizontal gene transfer which would not represent a concern for its use in the food chain.

The present study provides novel information about the use of LAB biofilm for the control of *E. coli* NCTC12900 colonization of inert surfaces. In particular, our objective was to identify LAB strains with high activity against *E. coli* O157:H7 NCTC 12900 and high biofilm formation activity to use them for the exclusion of EHEC on an inert surface at low temperature. *Lact. plantarum* CRL 1075 managed to inhibit EHEC, formed biofilm at 30°C and 10°C and reduced significantly the ability of the EHEC strain to form biofilm without disturbing its own biomass. Moreover, its absence of acquired antibiotic resistance, lead us to propose *Lact. plantarum* CRL1075 as a possible candidate for further studies oriented to its use as a bioprotective tool against EHEC in the food chain. The obtained results are encouraging since LAB strains from food could be used to reduce the incidence of pathogenic bacteria in food-processing facilities and/or meat display coolers. Additional *in situ* and technological studies are in progress to evaluate different possibilities of application of this bioprotective strategy.

MATERIALS AND METHODS

Bacterial strains and growth conditions

One hundred LAB strains isolated from food, belonging to the CERELA culture collection, were used in this study. Strains were stored at -20°C in milk yeast extract (10% w/v skim milk, 0.5% w/v yeast extract and 1% glycerol) and cultivated in MRS (de Man, Rogosa and Sharpe) broth (De Man et al. 1960) (Britania, Buenos Aires, Argentina) at 30°C or 37°C for 16-18 h. *E. coli* O157:H7 NCTC12900 (National Type Culture Collection, Colindale, London) was used as pathogen model for EHEC O157:H7 serotype. *E. coli*

O157:H7 NCTC12900 does not produce neither enterotoxins Stx1 nor Stx2 (Dibb-Fuller *et al.* 2001; Best *et al.* 2003). This strain was stored at -80°C in LB (Luria Bertani) medium in the presence of 20% glycerol as cryo-protectant. To obtain fresh cultures, the strain was transferred twice in LB broth and incubated at 37°C for 9 h, and for 16 h in the second transfer.

EHEC growth inhibition assay

The antagonistic potential of the 100 LAB strains against *E. coli* NCTC 12900 was tested using the agar diffusion assay according to Orihuel et al. (2018) with some modifications. To investigate the possible mechanisms of EHEC inhibition, for each LAB strain, different fractions from overnight (ON) liquid cultures in MRS medium were prepared: 1) concentrated suspensions of cells, i.e., cells that were recovered from ON cultures, washed and suspended in physiological solution (spot #1); 2) cells and associated extracellular components, i.e., the pure ON cultures (spot #2); 3) cell-free supernatants from ON cultures heated for 5 min at 97°C (to evaluate the potential inhibition of acid and heat stable extracellular compounds i.e., bacteriocins) (spot #3); 4) Idem to spot #3 and neutralized to pH 7.0 with 1 mol l^{-1} NaOH, (to neutralize produced acids) (spot #4) . In addition to the different fractions, 4% lactic acid was used as control of the acid effect (spot #5) (Saavedra *et al.* 2003).

For each LAB strain, 5 μl of each fraction were spotted onto MRS agar plates and allowed to dry. Then, a lawn of *E. coli* O157:H7 NCTC 12900 was generated in every spotted MRS plate by pouring 10 ml of soft LB agar (0.7%) containing EHEC cells from an ON culture (1/100 dilution). Plates were incubated at 30°C overnight and then analyzed for the appearance of inhibition zones over the spots. The anti-EHEC activity scores were assigned according to the diameter of inhibition zone: Negative (-) for inhibition halos ≤1.0 mm; Intermediate (+) for inhibition halos of 1.1–3.9 mm and High (++) for inhibition halos ≥4.0 mm.

This test was performed in independent triplicates and the means of the halos were calculated.

Biofilm Assays

The ability of LAB strains to form biofilms was evaluated according to the procedure described by Lebeer et al. (2007) with minor modifications. Briefly, 200 μl of each bacterial suspension in tMRS (de Man Rogosa and Sharpe without tween 80), adjusted to OD_{540} of 0.2, were added to individual wells in polystyrene 96-well microplates and incubated statically for 48 h at both 30°C and 10°C. In the latter case, an initial adherence of 8 h at room temperature was carried out. Every 24 h, the tMRS medium was replaced. After 48 h of incubation, supernatants were removed and wells were rinsed with PBS (phosphate buffer solution) to eliminate non-adherent cells. Then, biofilms on the surface of the wells were stained with 0.1%

crystal violet (CV) solution for 10 min. Excess of CV was removed by carefully washing the wells with distilled water. Plates were allowed to dry for 1 h at 60 °C. The CV bound to the biofilm biomass in each well was then extracted and solubilized by adding 200 μl of 30% (v/v) anhydrous acetic acid. The absorbance (A) at 570 nm of the resulting CV solutions was determined by using a microplate reader (Microplate reader, Bio-Rad, Hercules; CA, USA). Uninoculated tMRS broth was used as negative control. The cut-off for the microtiter-plate test (A_{COff}) was defined as three standard deviations above the mean of the negative control, according to Stepanović et al. (2000). Based on the values of A at 570 nm, the strains were classified as: non-biofilm producers ($A \leq A_{COff}$), weak ($A_{COff} < A \leq 2 \times A_{COff}$), moderate ($2 \times A_{COff} < A \leq 4 \times A_{COff}$) or strong biofilm producers ($4 \times A_{COff} < A$). Each assay was performed in triplicates with four technical repetitions. Results were expressed as the mean with its respective standard error (SE).

Meat-based medium

A meat-based broth that closely reproduces the meat composition was used as culture medium for exclusion assays. The meat-based broth was prepared as previously described (Fadda *et al*. 1998) with minor modifications. Briefly, 10 g of bovine semimembranosus muscle was homogenized with 100 mL of deionized water for 8 min in a Stomacher 400 blender (Stomacher, London, UK). After centrifugation of the homogenate (14,000 x g, 20 min at 4°C), the supernatant containing sarcoplasmic proteins and other soluble compounds was filtered through Whatman paper, filter-sterilized through a 0.22 µm-pore-size filter (Steritop GP, Biopore, Buenos Aires, Argentina) and supplemented with 0.5 % glucose. The sterility of the system was confirmed by plating in Plate Count Agar (PCA).

Exclusion assays at 10°C in a meat-based medium

The ability of pre-established LAB biofilms to prevent or inhibit the adhesion and biofilm formation of *E. coli* O157:H7 NCTC12900 on polystyrene microplates was evaluated as follows. For each LAB strain, cells derived from ON cultures in tMRS were transferred to the meat-based broth to achieve 10^7-10^8 CFU/ml. Adjusted cell suspensions were then seeded in individual wells. Microplates were first incubated at room temperature for 12 h to allow cells to attach to the surface of the wells and then incubated at 10°C for 36 h (48 h in total). Thereafter, supernatants were discarded and a suspension of *E. coli* NCTC12900 cells in meat-based medium (10^7-10^8 CFU/ml) was added to each well containing the LAB pre-formed biofilm. Microplates were incubated at 10°C for 24 h. After this incubation, wells were carefully washed twice with PBS and then individually scraped to remove all the biofilm biomass. Bacterial cells recovered from each well were suspended and adjusted in decimal dilutions, which were then plated on MRS and LB agar plates to determine the number of viable cells for LAB and *E. coli* O157:H7, respectively. The plates were incubated at 30°C for 24-48 h. Then, for each strain, the number of colonies on the plates was determined. Results were expressed as log CFU/cm^2, where cm^2 corresponds to the surface of the well. Biofilm formation of *E. coli* NCTC 12900 in the absence of LAB was used as control. Biofilm formation of LAB strains in the absence of EHEC was also used as control.

These experiments were carried out in triplicates, with duplicate samples per trial, and results were expressed as the mean and SE. The statistical significance of the differences associated to treatments was analyzed using one-way analysis of variance and Dunnet or Tukey test. A p value < 0.05 indicates statistical significance.

Antibiotics Susceptibility

Minimum inhibitory concentration (MIC) of eight antibiotics of clinical and veterinary importance against selected LAB strains were determined using the broth micro-dilution method set by ISO 10932/IDF 223 standard (Federation 2010). All assays were carried out in sterile 96-well microplates containing appropriate concentrations of antibiotics as indicated by the ISO/IDF protocol. The antibiotics used were selected by the European Food Safety Authority recommendations (EFSA 2012): ampicillin (AMP); chloramphenicol (CMP); tetracycline (TET), erythromycin (ERY), gentamicin (GEN), kanamycin (KAN), clindamycin (CLI) and streptomycin (STR) (Sigma-Aldrich, St. Louis, MO, USA). Plates were incubated at 30°C for 48 h. MIC were recorded as the lowest concentration of an antimicrobial agent at which visible growth was inhibited. The results were interpreted according to the breakpoints defined by EFSA (2012). The strains were categorized as susceptible when the MIC value was equal or lower than the cut-off value established by EFSA or resistant when the MIC value was higher than the cut-off value. Strains displaying growth until the cut-off value were subsequently analysed for the presence of antibiotic resistance genes. Total genomic DNA was extracted from the strains according to (Pospiech and Neumann 1995) modified protocol. The presence of TET, AMP and KAN resistance genes in resistant isolates was investigated by PCR (Polymerase chain reaction) using the specific primers (Table S1). Positive amplicons were purified and sequenced. The obtained sequences were compared with those in GenBank.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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Author contribution statement

CL: Designed the study, conducted the experiments, analyzed the results, contributed in the discussion and wrote the paper. CN and RC: Contributed in the experiments and in discussion of the paper. VMI: Contributed in the experiments and in the results analysis. SDO: contributed in the discussion and wrote the paper; YO: supervised the experiments, contributed in the discussion and wrote the paper. FS: conceived the idea for the project, coordinate the study, supervised the experiments, analyzed data, wrote the paper and participated in funding acquisition. All authors read and approved the manuscript.

Figure legends

Fig. 1 Biofilm-forming capacity of antagonistic LAB strains on polystyrene surfaces at 10ºC for 48 h. Strains were classified as strong, moderate, weak or no biofilm producers. Each bar represents the mean value of at least three independent experiments performed in quadruplicates. Asterisks indicate strains with the highest absorbance values at both temperatures. Error bars represent standard error.

Fig. 2 Exclusion assays at 10°C in a meat-based medium. EHEC NCTC12900 challenged with pre-established biofilms of LAB in a meat-based medium at 10°C for 24 h on polystyrene microplates. a) EHEC cells (Ec) counting (log CFU/cm^2) in absence (control Ec) or presence of *Lact. plantarum* (Lp) CRL 1075, Lp CRL 1482, Lp CRL 683, *Ped. pentosaceus* (Pp) CRL 2145, Pp CRL 908 biofilms. b) LAB cell counting (log CFU/cm^2) after biofilm growth either alone or subsequently challenged with EHEC cells. The squares represent the mean value and the dots represent each independent experiment value. One-way analysis of variance and Dunnet or Tukey test was applied. Different letters indicate statistically significant differences between the groups (p<0.05).

Supporting information

Table S1. Primers used to amplify transferable antibiotic resistance genes by PCR