ORIGINAL ARTICLE

Biological control of foodborne pathogens by lactic acid bacteria: A focus on juice processing industries

María Clara Tarifa[a,b], María del Rosario Agustín[c,d], Lorena Inés Brugnoni[c,d,*]

[a] *Universidad Nacional de Río Negro, CIT Río Negro, Río Negro, Argentina*
[b] *Centro de Investigaciones y Transferencia de Río Negro, CIT Río Negro (UNRN-CONICET), 8336 Villa Regina, Argentina*
[c] *Instituto de Ciencias Biológicas y Biomédicas del Sur, INBIOSUR (CONICET-UNS), 8000 Bahía Blanca, Argentina*
[d] *Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur (UNS), Argentina*

Abstract The use of lactic acid bacteria (LAB) in foods as biocontrol agents against food-borne pathogens has become increasingly known. Under the premise that controlling the adhesion of microorganisms to food contact surfaces is an essential step for meeting the goals of food processing, the aim of this work was to investigate the inhibitory and anti-biofilm effectiveness of *Lactobacillus rhamnosus* GG (ATCC 53103) and *Lactobacillus casei* (ATCC 393) against *Escherichia coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes*. *Lactobacillus* strains (10^8 CFU/ml) and pathogens (10^4 CFU/ml) were evaluated to monitor LAB anti-adhesive and antibiofilm effect, in two main scenarios: (i) co-adhesion and (ii) pathogen incorporation to stainless steel surfaces with a protective biofilm of *Lactobacillus* cells. In (i) the predominant effect was observed in *L. rhamnosus* against *S. enterica* and *L. monocytogenes*, whereas in (ii) both LAB significantly reduced the number of pathogenic adherent cells. The effect of pre-established LAB biofilms was more successful in displacing the three pathogens than when they were evaluated under co-adhesion. These findings show that both LAB can be considered good candidates to prevent or inhibit the adhesion and colonization of *L. monocytogenes*, *S. enterica* and *E. coli* O157:H7 on surfaces and conditions of relevance for juice processing industries, offering alternatives for improving the safety and quality of fruit-based products.
© 2023 Asociación Argentina de Microbiología. Published by Elsevier España, S.L.U. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

---

* Corresponding author.
 E-mail address: brugnoni@uns.edu.ar (L.I. Brugnoni).

Control biológico de patógenos de transmisión alimentaria mediante bacterias ácido lácticas: un enfoque en la industria elaboradora de jugos de frutas

Resumen Existe un creciente interés en el uso de bacterias ácido lácticas (BAL) como agentes de biocontrol frente a patógenos de transmisión alimentaria. Bajo la premisa de que el control de la adhesión de microorganismos a superficies de contacto con alimentos es el paso esencial para evitar su contaminación, el objetivo de este trabajo fue investigar la efectividad inhibitoria y antibiofilm de *Lactobacillus rhamnosus* GG (ATCC 53103) y *Lactobacillus casei* (ATCC 393) frente a *Escherichia coli* O157:H7, *Salmonella enterica* y *Listeria monocytogenes*. A fin de cumplir con el objetivo propuesto, las cepas de *Lactobacillus* (10^8 UFC/ml) y los patógenos (10^4 UFC/ml) se ensayaron en 2 escenarios: (1) coadhesión, y (2) incorporación de los patógenos a las superficies de acero inoxidable con un biofilm preformado de *Lactobacillus*. En (1), el efecto predominante se observó con *L. rhamnosus* frente a *S. enterica* y *L. monocytogenes*, mientras que en (2), ambas BAL redujeron significativamente el número de células patógenas adheridas. En función de estos resultados, concluimos que el efecto de un biofilm preformado de ambas BAL fue más exitoso en el desplazamiento de los 3 patógenos que en coadhesión. Ambas BAL pueden considerarse buenas candidatas para mitigar la adhesión y colonización de *L. monocytogenes*, *S. enterica* y *E. coli* O157:H7 en superficies en condiciones de relevancia para la industria procesadora de jugos, y, de esta manera, ofrecer alternativas para mejorar la seguridad y calidad de los alimentos a base de frutas.

Introduction

Fruits and fruit juices have been traditionally assumed to be low risk foods due to their acidic pH (~2.0 and 4.5); nonetheless, they have been recognized as ''emerging vehicles'' for foodborne illnesses caused by human pathogens such as *Escherichia coli* O157:H7, *Salmonella* spp., and *Listeria monocytogenes*[14]. *Salmonella enterica* and *E. coli* O157:H7 outbreaks have been previously associated with the consumption of apple and citrus juices[15], whereas other fruits such as mango, papaya and cantaloupe were responsible for foodborne outbreaks caused by *Salmonella* and *L. monocytogenes*[9]. Concentrated and reconstituted juices together with frozen puree or pulps have also been associated with foodborne pathogens such as *E. coli* O157:H7, *S. enterica* and *L. monocytogenes*[30]. They represent one of the major causes of foodborne outbreaks from food itself or, as result of biofilm formation, by cross-contamination from colonized food-related environments[16]. Indeed, Bridier et al.[6], reported, in a survey carried out in France, that *ca.* 60% of food-borne infections occurred as a result of microbial transfer from equipment and surfaces to processed foods, reinforcing the need for control.

With mechanical and process automation, the surfaces are in repeated contact with raw juice, thus increasing the opportunities for pathogenic microorganisms to transfer and attach, leading to biofilm formation. In this regard, *L. monocytogenes* is commonly found in food-processing environments, residing in so-called refuge sites such as cracks, worn equipment and in hard to reach places such as complex machinery that may be subjected to suboptimal disinfection concentrations, allowing them to survive and possibly adapt to cleaning and sanitation treatments[7]. Mendonça et al.[28],

also demonstrated that *E. coli* O157:H7 has the potential to form biofilms on different surfaces (*e.g.* polystyrene, polytetrafluoroethylene, glass and stainless-steel) commonly used in food-processing plants, and in the case of *Salmonella* spp., common sites for their presence are, for example, filling or packaging equipment, floor drains, walls, cooling pipes and conveyors[34]. The persistence and survival of these pathogens on food-contact surfaces represent a potential chronic source of microbial contamination, threatening their safety and quality besides hurting the commercial aspects of production[5,21,37].

Food companies traditionally rely on cost-effective easy to use chemical methods, such as sodium hydroxide or sodium hypochlorite solutions for the elimination or prevention of biofilm development along processing plant pipes and surfaces. However, the development of biofilms on some food industry structures cannot be controlled with these cost-effective methods[47,48]. It is interesting to note that decades of using sanitizers in the food industry may be one of the main driving forces regarding the development of antibiotic resistance in bacteria and its dissemination to pathogens[16]. Biofilms in the food industry have been suggested as hot-spots for plasmid transfers, including antibiotic multi-resistant plasmids. Moreover, there is a growing demand and awareness of consumers of food quality and safety, as well as environmental concerns, which have led to the search of potential alternative hurdle technologies to control biofilms independently of the traditional chemical approach that regulates the protocols implemented nowadays.

A promising strategy toward biofilm prevention and control is the use of lactic acid bacteria (LAB) which, through the colonization of hard surfaces and based on the

competitive exclusion principle, prevent the proliferation of other bacterial species[13]. This concept, in which the potential of LAB as bioprotective agents against pathogenic microorganisms is highlighted, has been designated as biocontrol[44]. For many centuries, microbial antagonism has been used in food processing to improve food safety and, in the last decade, several studies have shown that anti-adhesive and anti-biofilm attributes for some LAB represent a potential counteracting approach to prevent pathogen implantation, without conferring any risks to consumers[31,38,39,42]. In this regard, food biocontrol with natural and microbiological compounds may be a satisfactory approach to solve economic losses not only due to microbial spoilage of raw materials and food products, but also to reduce the incidence of foodborne illness[12]. However, one of the main challenges that need to be contemplated when using LAB as a means of biocontrol in food or food-contact surfaces is to ensure their growth and survival in the food environment where they must exert their effect. Most of the research performed up to now, has been carried out under *in vitro* optimal conditions; however, to develop a relevant biocontrol approach, it is mandatory to take into account the *in situ* prevailing conditions that influence the performance of antagonistic bacteria and their effectiveness, such as target microorganism, growing state and nature of the food matrix.

Since the application of LAB as biocontrol agents in food-contact surfaces of fruit juices processing industries has not yet been fully developed, the main objective pursued here was to test the ability of *Lactobacillus casei* ATCC 393 and *Lactobacillus rhamnosus* ATCC 53103 to mitigate *E. coli* O157:H7, *S. enterica* and *L. monocytogenes* during colonization and biofilm formation. A key factor when testing situations related to food industry, is to use procedures that are relevant to the environment in which the microorganisms will be exposed, and, therefore, in the present study the following factors were taken into account to approximate to a real model: (1) apple and grape juice as culture medium; (2) incubation of cultures at 25 °C, which is usual in juice processing plants and (3) stainless steel AISI 304 used as food contact material.

Materials and methods

Food matrix

Apple (Ap, 12°Brix) and grape (G, 23°Brix) juice without pulp, additives, or preservatives, produced by local producers of the Alto Valle de Río Negro (Patagonia, Argentina) were used as food matrices. The 12°Brix clarified Ap juice used was prepared from 72°Brix concentrated Ap juice, maintaining the specifications of the original matrix. Before use, each juice was sterilized by microfiltration with 0.45 μm pore size membranes (Metricel®Grid, Gelman-Sciences, Ann Arbor, MI, USA) and Brix levels were determined with a digital WYA Abbe refractometer.

Microorganisms and culture conditions

Three bacterial pathogens were used: *E. coli* O157:H7 EDL 933 (ATCC 700927) Stx1 and Stx2 producer;

L. monocytogenes (ATCC 7644) serovar 1/2c and *S. enterica* serotype Enteriditis isolated from poultry at the Laboratorio de Microbiología Industrial y de los Alimentos of the Universidad Nacional del Sur (Buenos Aires, Argentina). The identification of *Salmonella* spp. was confirmed by using conventional standard biochemical and serological tests at the reference laboratory Instituto Dr. Carlos G. Malbrán (Buenos Aires, Argentina). LAB used included *L. casei* (ATCC 393) and *L. rhamnosus* (ATCC 53103), both purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA).

Stock cultures of *L. rhamnosus* and *L. casei* stored at −70 °C in 20% (v/v) glycerol were suspended in De Man, Rogosa and Sharpe broth (MRS, Biokar Diagnostics, Beauvais, France) and incubated for 24 h at 37 °C, while pathogens were grown in Tryptic Soy Broth (TSB, Difco, Detroit, MI, USA) for 24 h at 37 °C. After incubation, suspensions were harvested by centrifugation at $5000 \times g$ for 10 min (Labofuge 200, Kendro, Hamburg, Germany) and cell pellets were washed twice with sterile Phosphate-Buffered Saline (PBS: 0.15 mol/l NaCl, 0.05 mol/l KH$_2$PO$_4$, 0.05 mol/l K$_2$HPO$_4$, pH 7.2) before re-suspension in the corresponding food matrix: Ap and G juice. For LAB, suspensions were adjusted by optical density (OD) at 600 nm to 0.250 (∼10^8 cells/ml) whereas for the pathogens, a working culture suspension of 10^4 CFU/ml was obtained by serially diluting an overnight culture.

Preparation of acid-adapted pathogens

To adhere to surfaces and proliferate, pathogen cells must first survive in the acidic environments of the food systems encountered. Thus, the use of acid-adapted cells better represents the ongoing reality rather than the use of healthy exponential growing cultures; to do so *E. coli* O157:H7, *S. enterica* and *L. monocytogenes* were acid-adapted using the method described in Tarifa et al.[41]. Briefly, pathogens were pre-adapted by their inoculation in Ap or G juice for 4 h at 25 °C, harvested by centrifugation at $5000 \times g$ for 10 min and resuspended in the corresponding juice to prepare the final working inoculum.

Surface pre-condition, adhesion tests and biofilm formation

Surface pre-condition
Stainless steel coupons (SSC) of 1 cm^2 area, type AISI-304 were used as abiotic surfaces for attachment assays. The experiments were performed in sterile polyvinyl chloride 24-well microtiter plates (JET BIOFIL, Argentina) with one sterile SSC per well.

Adhesion tests
The potential of LAB to control the adherence of pathogens to SS and mitigate their ability to develop biofilms was evaluated by competition and exclusion. To this purpose, the combinations tested were: (i) *L. casei* or *L. rhamnosus* + *E. coli* O157:H7; (ii) *L. casei* or *L. rhamnosus* + *S. enterica*; (iii) *L. casei* or *L. rhamnosus* + *L. monocytogenes*.

For the competition assays, *L. casei* and *L. rhamnosus* were co-cultured with the three pathogens separately. To do

so, standardized suspensions of each microbial group (LAB and pathogens) were mixed in a 1:1 ratio, poured into each well containing a SSC and incubated at 25 °C for 24 h. On the other hand, for the exclusion assays, a 24 h preformed biofilm of *L. casei* and *L. rhamnosus* was grown on the SSCs, then the supernatant was carefully discarded and surfaces followed two rinsing steps with PBS to remove the loosely attached cells and leave the adherent ones. Afterwards, the adjusted suspensions of each pathogenic bacterium were separately put in contact with the pre-adhered *Lactobacillus* cells and incubated for another 24 h at 25 °C. Meanwhile, the attachment of LAB and pathogens in Ap and G juice was tested independently as control. *E. coli* O157:H7, *S. enterica* and *L. monocytogenes* were put in contact with SSC for 24 h, while *L. casei* and *L. rhamnosus* for 24 and 48 h. All the assays described were carried out under static conditions at 25 °C.

Quantification of cells

For each condition, coupons were carefully removed from the wells using sterile forceps and rinsed by immersion for 2 min in 5 ml of PBS with gentle agitation to remove the loosely attached cells and then properly processed to accomplish count procedures. Before enumeration, SSCs were subjected to two sequential detachment treatments according to Lindsay and Von Holy[25], and Trampuz et al.[45], with some modifications. First, the surfaces were placed in test tubes containing PBS and glass beads (5 mm diameter, up to complete coverage) and sonicated (Digital Ultrasonic Cleaner, PS-10A, Arcano, China) for 2 min at 20 °C and 40 kHz. Next, the coupons were vortexed for 2 min followed by plating the proper decimal dilutions in MRS agar (Biokar, France) in the case of LAB, Eosin Metil Blue agar (EMB, Britania, Argentina) for *E. coli* O157:H7, gelose Oxford agar (Biokar Diagnostics, Beauvais, France) with selective supplementation (Oxford BS00308, Biokar Diagnostics, Beauvais, France) for *L. monocytogenes* and Bismuth Sulfite Agar (BSA, Merck KGaA, Germany) for *S. enterica*, followed incubation for 24–48 h at 37 °C. Results were expressed as log CFU of adhered cells per cm^2 (Log_{10} CFU/cm^2).

To ensure complete removal of the bacterial cells, each SSC was stained with sterile fluorescein diacetate (FDA) acetonic solution in 0.1 mol/l PBS (0.04% v/v), pH 7.5. After 90 min agitation in darkness, the coupons were rinsed twice with sterile distilled water, air-dried, and observed under an epifluorescence microscope (Olympus BX 51, NY, USA) using a 1009 oil-immersion objective, blue excitation U-MWB2.

Scanning electron microscopy (SEM)

To determine the adhesion patterns of *Lactobacillus* cells on SS, samples were fixed with glutaraldehyde (2.5%) in phosphate buffer (0.1 mol/l, pH 7.2); washed three times with the same buffer and dehydrated by critical point drying (E3000, Polaron Instruments, Hatfield, PA, USA). Samples were gold-coated (300 Å) in a Pelco Model 3 Sputter Coater 91000 metal evaporator and viewed under a scanning electronic microscope (LEO EVO 40, Cambridge, UK) at 7.0 kV acceleration voltage.

Physicochemical analysis

In order to test the potential effect that the *Lactobacillus* strains proposed as biocontrol agents could have on the food matrix, the main physicochemical determinations associated to their impact on juices were measured: pH, °Brix, titratable acidity (TA) and turbidity. To this purpose, the system described in Tarifa et al.[40], was used with some modifications. Briefly, SS disks (50 mm diameter, 0.5 mm thickness; AISI 304, food grade) were placed in beakers and, to allow microbial attachment, were filled with an adjusted suspension of each LAB prepared as described in ''Microorganisms and culture conditions'' section up to a final working volume of 250 ml per beaker system. Initially, *Lactobacillus* cells were allowed to deposit on the SS disks for 24, 120 and 168 h at 25 °C, then the surfaces containing the protective LAB biofilm were carefully moved to another sterile beaker, filled with the same working volume of fresh juice and incubated for 48 h, time after which the juice was taken and analyzed. pH was determined using a Hanna HI5521 digital pH meter (Hanna Instruments SL, Gipuzkoa, Spain) calibrated with buffers at pH 4.0 (Merck, Darmstadt, Germany) and 7.0 (Cicarelli, Santa Fe, Argentina). TA was performed using the AOAC method No 16.023[3] by titration with 0.1 N NaOH solutions and expressed as percentage of lactic acid[3]. Brix levels were determined with a digital WYA Abbe refractometer as previously mentioned and turbidity was measured using a Velp Scientifica TB1 portable turbidimeter and expressed as nephelometric turbidity units (NTU).

Statistical analysis

Each measurement was performed in triplicate with independent bacterial cultures in each case. Counts were converted to decimal logarithmic values (log CFU) to nearly match the assumption of a normal distribution and the results were expressed as in mean with standard deviation. In order to verify the difference between samples, a variance analysis (one-way ANOVA) was applied followed by the Tukey test or *t*-test at a significant level of 5% ($p < 0.05$). All statistical analyses were performed using GraphPad InStat (Software, Inc. 2003).

Results and discussion

Bacterial adhesion in monoculture

Initially, the adhesion potential to SS of each of the species separately (*L. casei*, *L. rhamnosus*, *E. coli* O157:H7, *S. enterica* and *L. monocytogenes*) was tested. The results indicated the following: (1) both types of microbial groups (LAB and pathogens) can survive in both types of juice under the conditions tested, (2) they can adhere to SS, with a stronger potential for LAB strains compared to pathogens and (3) the adhesion degree depended on the type of juice being greater in G than in Ap juice.

Lactobacillus casei was the species with the highest population of adherent cells with viable counts in Ap juice of 6.69 ± 0.12 log CFU/cm^2 after 24 h adhesion and
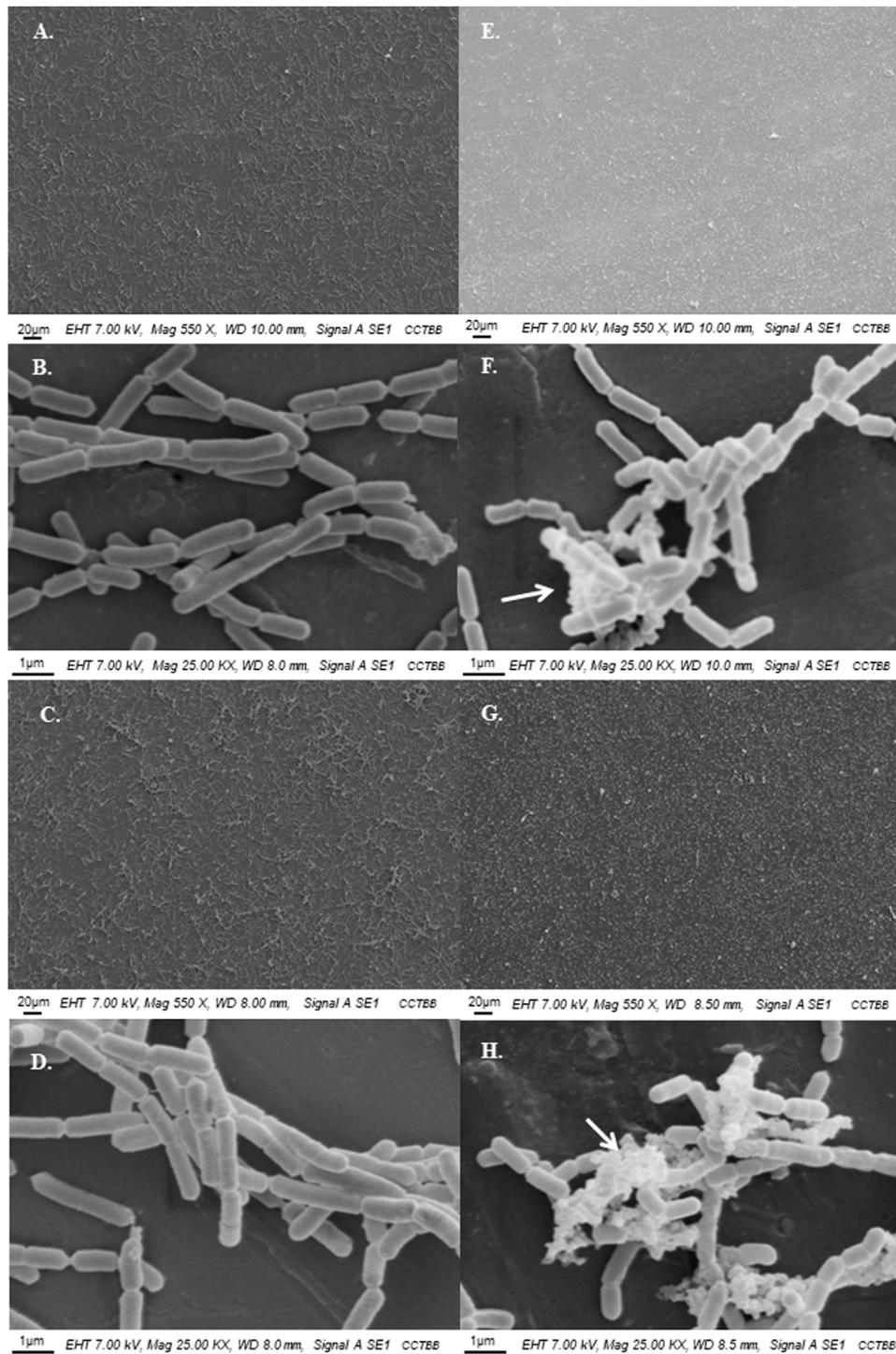
Figure 1 SEM observations of the attached cells of lactic acid bacteria on stainless steel AISI 304 incubated at 25 °C in 12°Brix apple juice. Left panel: *L. rhamnosus* ATCC 53103 and right panel: *L. casei* ATCC 393; (A, B, E, and F) 24 h, (C, D, G, and H) 48 h. Arrows show the presence of EPS.

6.95 ± 0.31 log CFU/cm² at 48 h *vs* 7.08 ± 0.06 log CFU/cm² and 7.48 ± 0.26 log CFU/cm² in G juice after 24 and 48 h adhesion, respectively. In the case of *L. rhamnosus*, counts ranged between 4.85 ± 0.69 log CFU/cm² and 5.71 ± 0.65 log CFU/cm² for 24 and 48 h in Ap juice, and in G juice between 6.51 ± 0.05 log CFU/cm² and 6.61 ± 0.21 log CFU/cm², at 24

and 48 h respectively. When analyzing their adhesion pattern by SEM (Fig. 1), the coverage potential could be confirmed with a homogeneous progression from 24 to 48 h. This fact, together with the presence of extracellular polymeric substances (EPS) in the case of *L. casei* (Figs. F and H), is a clear indicator of the good adhesive capacity of both

Lactobacillus species, thus suggesting that they could be good candidates to form protective biofilms on SS under juice production environments. Our findings support the hypothesis previously reported by Falagas and Makris[13], in which they propose using non-pathogenic microorganisms as part of daily cleaning products to lower the incidence of pathogenic microorganisms, and also that reported by Toushik et al.[44], which states the promising anti-biofilm properties of two LAB to control foodborne pathogens in the seafood safety research sector. In fact, the present study focuses on an unattended sector such as juice processing industries, seeking to identify a potential biocontrol strategy capable of reducing juice perishability by inhibiting food-borne pathogens.

In terms of the three pathogens tested, the highest adhesion potential was observed for *S. enterica* (2.81 ± 0.47 log CFU/cm^2 [Ap]-4.53 ± 0.45 log CFU/cm^2 [G]) followed by *L. monocytogenes* (2.48 ± 0.74 log CFU/cm^2 [Ap]-4.33 ± 0.19 log CFU/cm^2 [G]) and *E. coli* O157:H7 (1.85 ± 0.21 log CFU/cm^2 [Ap]-3.89 ± 0.04 log CFU/cm^2 [G]). SEM analysis of the attachment capacity and adhesion patterns of the three pathogens has been previously described in studies conducted by the group[1,41]. As evidenced by the results, *E. coli*, *S. enterica* and *L. monocytogenes* can, not only tolerate the adverse conditions imposed, for example, by the acidic food matrix, deploying mechanisms of resistance to stress[18,33], but also adhere to SS surfaces, implying a permanent and persistent risk of cross-contamination of products in contact with them. These results reinforce the ones achieved in previous reports[1,40,41] of the group, where, combined, they establish the capacity of *E. coli* O157:H7, *S. enterica* and *L. monocytogenes* to adapt after short periods of time to moderate acidic conditions, such as the ones imposed by Ap juice, improving their survival in the food matrix. Even though, Just and Daeschel[23], reported survival periods for *E. coli* O157:H7 and *Salmonella* spp. of up to 16 d in grape juice, a small amount of information is available concerning their adhesion and behavior on SS under conditions of relevance for juice-producing industries. What is more, to the best of our knowledge this is among the first and latest reports done on the survival and adaptation of these bacterial pathogens in G juice under simulated industrial conditions. The survival, adhesion and resistance reported in this work in Ap and G juice support the possibility that fresh juices without further processing could act as a vector for pathogens and, thus, the urge for their control in processing facilities where they are produced.

According to González-Pérez et al.[20], who stated that the adaptation of bacteria to different niches often confers particular characteristics, the experimental results show that the conditions used (food matrix, surface and temperature) influenced the adhesion potential of the studied species, especially for pathogens, which are the target microorganisms. In view of the above, the importance of studying the dynamic interactions between the different groups of bacteria and the surfaces in each specific food processing environment is highlighted in order to provide more effective measures.

Effect of probiotic biofilms on pathogen sessile growth: competition (co-culture) and exclusion assays (preformed LAB biofilm)

In order to evaluate the effect that LAB biofilms could have on the initial attachment steps of pathogens, evidence was provided on the growth of *L. monocytogenes*, *E. coli* O157:H7 and *S. enterica* in their sessile form under different circumstances such as competition (co-adhesion) and exclusion (pre-formed biofilm). As shown in Table 1, *L. casei* and *L. rhamnosus* were able to inhibit *E. coli* O157:H7, *S. enterica* and *L. monocytogenes* adhesion to SS in Ap juice to below the detectable limits under competition and exclusion. Similar to our study, Gómez et al.[19], used *in situ* biofilms formed by potential probiotic LAB strains isolated from Brazilian charqui, goat cheese, ripened cheese and salami to inhibit pathogenic growth and found total inhibition of *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* Typhimurium biofilm formation after 24, 48, and 72 h of exposure using *L. lactis* 368, *L. curvatus* MBSa3 and *L. sakei* MBSa1 arguing that exclusion mechanisms could be responsible for the final result. Similarly, Woo and Ahn[46], have shown that probiotic *Lactobacillus* strains (*L. acidophilus* KACC 12419, *L. paracasei* KACC 12427, *L. casei* KACC 12413, and *L. rhamnosus* KACC 11953) can effectively reduce biofilm formation by the foodborne pathogens *S. enterica* subsp. *enterica* serovar Typhimurium and *L. monocytogenes*, through competition, exclusion, and displacement mechanisms; even though, Rendueles and Ghigo[36], reported that the production of inhibitory compounds may also act by delaying or inhibiting their growth. Moreover, Pérez-Ibarreche et al.[31], using pre-colonized SS and polytetrafluoroethylene surfaces by *L. sakei* CRL1862 prevented the attachment of *L. monocytogenes* FBUNT cells, an Argentinian clinical isolate serotype 4b.

In the case of G juice, complete elimination or at least significant reductions of *E. coli* and *L. monocytogenes* counts were achieved for both LAB in competition and exclusion assays. In addition, *S. enterica* evidenced a significant decrease when faced with *L. casei* pre-established biofilm. In contrast, both LAB failed to reduce the number of *S. enterica* sessile cells in competition assays (co-adhesion). Similarly, Kamal et al.[24], observed that the addition of *L. rhamnosus* to yoghurt failed to inhibit *S. typhimurium* in co-culture until 72 h of cold storage. Alvarez-Ordóñez et al.[2], concluded that this behavior can be attributed to the phenotypic acid tolerance response of *Salmonella* spp. Several investigations have demonstrated that *Salmonella* spp. can survive in acidic foods at low pH values for long-term periods. Similar to the considerations taken into account by Cisneros et al.[11], when analyzing the exclusion potential of certain LAB strains against enterohemorrhagic *E. coli* (EHEC) in a meat-based medium, the number of pathogenic cells attached, for example, to a stainless steel pipeline in a real scenario in juice processing industries would be significantly lower than the inoculum used, and therefore would be appropriate to hypothesize that LAB will be able to completely exclude *E. coli*, *S. enterica* and *L. monocytogenes*.

Table 1 *L. casei* ATCC 393 and *L. rhamnosus* ATCC 53103 inhibition effect toward *E. coli* O157:H7, *S. enterica,* and *L. monocytogenes* adhesion and biofilm formation on stainless steel after co-adhesion in a 1:1 ratio, and incorporation to pre-adhered *Lactobacillus* spp. cells using apple and grape juice as food matrix

Pathogen	Juice	Control	Adhesion (log CFU/cm^2)			
			Lactobacillus rhamnosus		*Lactobacillus casei*	
			Co-adhesion	Preformed	Co-adhesion	Preformed
Escherichia coli O157:H7	Apple	1.85 ± 0.21	ND	ND	ND	ND
	Grape	3.89 ± 0.04	2.94 ± 0.34[**]	ND	2.75 ± 0.21[**]	ND
Salmonella enterica	Apple	2.81 ± 0.47	ND	ND	ND	ND
	Grape	4.53 ± 0.45	3.93 ± 0.28[ns]	3.96 ± 0.01[ns]	4.69 ± 0.06[ns]	ND
Listeria monocytogenes	Apple	2.48 ± 0.74	ND	ND	ND	ND
	Grape	4.33 ± 0.19	ND	ND	ND	ND

Results are expressed as log CFU/cm^2 ± SD. Significant differences are expressed through the following nomenclature *$p < 0.05$, **$p < 0.01$, and [ns]not significant. ND: not detected, below limit of detection.

Table 2 Physicochemical parameters of apple juice after exposure to *L. casei* ATCC 393 and *L. rhamnosus* ATCC 53103 biofilms

Condition	Contact time (h)	pH	Brix	Turbidity (NTU)	TA (% lactic acid)
Control		3.59 ± 0.05	10.43 ± 0.60	0.23 ± 0.12	3.22 ± 0.09
L. casei	24	3.65 ± 0.09	10.20 ± 0.42	2.95 ± 1.52	2.37 ± 0.77
	120	3.45 ± 0.21	10.50 ± 0.71	0.43 ± 0.17	4.27 ± 1.30
	168	3.30 ± 0.23	10.01 ± 0.27	0.50 ± 0.18	3.59 ± 0.85
L. rhamnosus	24	3.62 ± 0.07	11.00 ± 0.71	0.92 ± 1.22	2.63 ± 1.39
	120	3.63 ± 0.12	10.50 ± 0.71	0.24 ± 0.27	3.98 ± 1.35
	168	3.53 ± 0.04	10.00 ± 0.41	0.49 ± 0.06	3.02 ± 0.38

TA: titratable acidity.

In fact, the results obtained suggest that *L. rhamnosus* and *L. casei* are able to restrain the adhesion and proliferation of the three pathogens tested in Ap. Nevertheless, as evidenced in the case of G juice, the food matrix is a factor that must be taken into consideration before selecting the biocontrol strategy to be used as it is a factor that proved to influence the final outcome. As observed in G juice, *L. casei* prevented the proliferation of pathogens whereas the effect of *L. rhamnosus* depended on the pathogenic species to which it was confronted. Furthermore, and in line with the results obtained in the present study, Hascoët et al.[22], and Toushik et al.[44], advocated two possible hypotheses regarding the effect that a LAB preformed biofilm could have on pathogens: (i) non-specific interaction (competition, displacement and exclusion) hypothesis where the pre-established biofilms of other microorganisms affect the implantation of the pathogen on the surface by occupying the available space; and (ii) specific competition hypothesis in which pre-existing microorganisms affect the development of the pathogen biofilm by competing for nutrients or producing anti-microbial substances such as bacteriocins, anti-quorum sensing compounds, and enzymes, among others. On the other hand, the inhibitory performance of LAB according to Arena et al.[4], could be due to the organic acids released by several lactobacilli that could also ascribed to or potentiate the effect over their anti-adhesive properties. Tejero-Sariñena et al.[43], observed that the growth-inhibiting

activity of different LAB, *i.e.*, strains belonging to *Lactobacillus*, against pathogens such as *S. typhimurium* and *E. coli* was attributed to a pH reduction and/or to the production of organic acids, including lactate and acetic acid.

Effect of LAB over juice physicochemical properties

Data from Table 2 compiles the results obtained after evaluating the potential effect that *L. casei* and *L. rhamnosus* had on the food matrix. The parameters tested were the ones mostly observed as indicators of spoilage by this type of microorganisms when exposed to juice: pH, °Brix, TA and turbidity. No significant differences ($p > 0.05$) were observed between controls and samples in contact with pre-adhered *Lactobacillus* cells. LAB species, such as lactobacilli, leuconostocs, and weissellas, have usually been associated with spoilage of fruit juices through the production of turbidity and excessive acidity[35]. On the contrary, no substantial changes to the food matrix were noted when using LAB. Indeed, LAB had aroused much interest as biocontrol agents on surfaces, packaging and as bioconservatives in the food itself[26,27]. The present study allowed to delve into the study of LAB as biocontrol agents against foodborne pathogens and their application as a ''green'' alternative in juice processing facilities, demonstrating an alternative growth and

adhesion control of pathogens using an ecofriendly strategy through microbial implementation.

Antimicrobial intervention technologies through the application of decontamination treatments or antimicrobial procedures for inhibition or reduction of microbial growth are gaining interest in order to reduce bacterial contamination[8]. Based on the data obtained in this study, *L. casei* and *L. rhamnosus* could be considered good candidates for applications as natural barriers or competitive-exclusion microorganisms to control *E. coli*, *S. enterica* and *L. monocytogenes* biofilm formation. The integral combination of factors such as target microorganism and nature of the food matrix clearly influences the performance that *Lactobacillus* strains have and thus might influence their effectiveness. In this sense, several studies conducted previously[10,17,29,32] have highlighted the role of food matrices in both biofilm formation and efficacy of biocontrol treatments reinforcing the need to a deeper study, especially in juice production lines where small amount of information is available about biocontrol.

Conclusions

This study highlights the potential that the implementation of a protective biofilm of LAB has to inhibit growth and adhesion of *E. coli* O157:H7, *S. enterica* and *L. monocytogenes* on surfaces commonly used in apple and grape juice production settings. Regardless of the type of juice used, *L. rhamnosus* and *L. casei* showed that can be considered good candidates for controlling the adhesion of these foodborne pathogen in juice-related industries, mainly when used in a protective biofilm form.

Even though both LAB showed that, under the conditions tested, they can be considered an ecofriendly alternative to mitigate the poor response that biofilms currently have to conventional control methods, further analyses should be performed to confirm that the application of these strains as potential biocontrol agents does not alter the sensory properties of the final products on an industrial scale.

Authors' contributions

Tarifa María Clara: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. Agustín María del Rosario: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. Brugnoni Lorena Inés: Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Funding acquisition.

Conflict of interest

The authors have no conflicts of interest to declare.

Acknowledgments

References

1. Agustín MdeR, Brugnoni LI. Multispecies biofilms between *Listeria monocytogenes* and *Listeria innocua* with resident microbiota isolated from apple juice processing equipment. J Food Saf. 2018;38:e12499.
2. Alvarez-Ordóñez A, Broussolle V, Colin P, Nguyen-The C, Prieto M. The adaptive response of bacterial food-borne pathogens in the environment, host and food: implications for food safety. Int J Food Microbiol. 2015;213:99–109.
3. AOAC – Association of Official Analytical Chemists. Official methods of analysis. Titratable acidity – ''Acidity-Official Final Action,'' section 16.023. 13th ed. Gaithersburg, MD, USA: AOAC International; 1980.
4. Arena MP, Silvain A, Normanno G, Grieco F, Drider D, Spano G, Fiocco D. Use of *Lactobacillus plantarum* strains as a bio-control strategy against food-borne pathogenic microorganisms. Front Microbiol. 2016;7:464.
5. Barbosa AAT, Mantovani HC, Jain S. Bacteriocins from lactic acid bacteria and their potential in the preservation of fruit products. Crit Rev Biotechnol. 2017;37:852–64.
6. Bridier A, Sanchez-Vizuete P, Guilbaud M, Piard JC, Naïtali M, Briandet R. Biofilm-associated persistence of food-borne pathogens. Food Microbiol. 2015;45:167–78.
7. Carpentier B, Cerf O. Persistence of *Listeria monocytogenes* in food industry equipment and premises. Int J Food Microbiol. 2011;145:1–8.
8. Castellano P, Pérez Ibarreche M, Blanco Massani M, Fontana C, Vignolo GM. Strategies for pathogen biocontrol using lactic acid bacteria and their metabolites: a focus on meat ecosystems and industrial environments. Microorganisms. 2017;5:38.
9. CDC. [Internet] List of selected multistate foodborne outbreaks notices since 2006. Available from: http://www.cdc.gov/foodsafety/outbreaks/multistate-outbreaks/outbreaks-list.html.
10. Chaitiemwong N, Hazeleger W, Beumer R. Inactivation of *Listeria monocytogenes* by disinfectants and bacteriophages in suspension and stainless steel carrier tests. J Food Prot. 2014;77:2012–20.
11. Cisneros L, Cattelan N, Villalba MI, Rodriguez C, Serra DO, Yantorno O, Fadda S. Lactic acid bacteria biofilms and their ability to mitigate *Escherichia coli* O157: H7 surface colonization. Lett Appl Microbiol. 2021;73:247–56.
12. Cizeikiene D, Juodeikiene G, Paskevicius A, Bartkiene E. Antimicrobial activity of lactic acid bacteria against pathogenic and spoilage microorganism isolated from food and their control in wheat bread. Food Control. 2013;31:539–45.
13. Falagas ME, Makris GC. Probiotic bacteria and biosurfactants for nosocomial infection control: a hypothesis. J Hosp Infect. 2009;71:301–6.
14. FDA. Hazard analysis and critical control point (HAACP): procedures for the safe and sanitary processing and importing of juice; final rule. Federal Register. 2001;66:6138–202.
15. Gabriel AA. Influences of simultaneous physicochemical stresses on injury and subsequent heat and acid resistances of *Salmonella Enteritidis* in apple juice. Food Control. 2013;31:28–34.
16. Galié S, García-Gutiérrez C, Miguélez EM, Villar CJ, Lombó F. Biofilms in the food industry: health aspects and control methods. Front Microbiol. 2018;9:898.
17. Ganegama Arachchi GJ, Cridge AG, Dias-Wanigasekera BM, Cruz CD, McIntyre L, Liu R, Flint S, Mutukumira A. Effectiveness of phages in the decontamination of *Listeria monocytogenes* adhered to clean stainless steel, stainless steel coated with fish protein, and as a biofilm. J Ind Microbiol Biotechnol. 2013;40:1105–16.
18. Giaouris E, Heir E, Desvaux M, Hébraud M, Møretrø T, Langsrud S, Doulgeraki A, Nychas G-J, Kačániová M, Czaczyk K, Ölmez H,

Simões M. Intra-and inter-species interactions within biofilms of important foodborne bacterial pathogens. Front Microbiol. 2015;6:841.

19. Gómez NC, Ramiro JM, Quecan BX, de Melo Franco BD. Use of potential probiotic lactic acid bacteria (LAB) biofilms for the control of *Listeria monocytogenes*, *Salmonella typhimurium*, and *Escherichia coli* O157: H7 biofilms formation. Front Microbiol. 2016;7:863.

20. González-Pérez C, Vargas-Arispuro I, Aispuro-Hernández E, Aguilar-Gil C, Aguirre-Guzmán Y, Castillo A, Hernandez-Mendoza A, Ayala-Zavala JF, Martinez-Tellez MA. Potential control of foodborne pathogenic bacteria by *Pediococcus pentosaceus* and *Lactobacillus graminis* isolated from fresh vegetables. Microbiol Biotechnol Lett. 2019;47:183–94.

21. Gray JA, Chandry PS, Kaur M, Kocharunchitt C, Bowman JP, Fox EM. Novel biocontrol methods for *Listeria monocytogenes* biofilms in food production facilities. Front Microbiol. 2018;9:605.

22. Hascoët AS, Ripolles-Avila C, Cervantes-Huamán BR, Rodríguez-Jerez JJ. In vitro preformed biofilms of *Bacillus safensis* inhibit the adhesion and subsequent development of *Listeria monocytogenes* on stainless-steel surfaces. Biomolecules. 2021;11:475.

23. Just J, Daeschel M. Antimicrobial effects of wine on *Escherichia coli* O157: H7 and *Salmonella typhimurium* in a model stomach system. J Food Sci. 2003;68:285–90.

24. Kamal RM, Alnakip ME, El Aal SFA, Bayoumi MA. Bio-controlling capability of probiotic strain *Lactobacillus rhamnosus* against some common foodborne pathogens in yoghurt. Int Dairy J. 2018;85:1–7.

25. Lindsay D, Von Holy A. Evaluation of dislodging methods for laboratory-grown bacterial biofilms. Food Microbiol. 1997;14:383–90.

26. Maillet A, Denojean P, Bouju-Albert A, Scaon E, Leuillet S, Dousset X, Jaffrès E, Combrisson J, Prévost H. Characterization of bacterial communities of cold-smoked salmon during storage. Foods. 2021;10:362.

27. Martinez RCR, Staliano CD, Vieira ADS, Villarreal MLM, Todorov SD, Saad SMI, de Melo Franco BDG. Bacteriocin production and inhibition of *Listeria monocytogenes* by *Lactobacillus sakei* subsp. sakei 2a in a potentially synbiotic cheese spread. Food Microbiol. 2015;48:143–52.

28. Mendonça RCS, Morelli AMF, Pereira JAM, de Carvalho MM, de Souza NL. Prediction of *Escherichia coli* O157: H7 adhesion and potential to form biofilm under experimental conditions. Food Cont. 2012;23:389–96.

29. Oliveira RPDS, Pinheiro R, Perego P, de Oliveira MN, Converti A. Effect of inulin on the growth and metabolism of a probiotic strain of *Lactobacillus rhamnosus* in co-culture with *Streptococcus thermophilus*. LWT. 2012;47:358–63.

30. Oyarzabal OA, Nogueira MC, Gombas DE. Survival of *Escherichia coli* O157: H7, *Listeria monocytogenes* and *Salmonella* in juice concentrates. J Food Prot. 2003;66:1595–8.

31. Pérez-Ibarreche M, Castellano P, Leclercq A, Vignolo G. Control of *Listeria monocytogenes* biofilms on industrial surfaces by the bacteriocin-producing *Lactobacillus sakei* CRL1862. FEMS Microbiol Lett. 2016;363:118.

32. Perricone M, Bevilacqua A, Altieri C, Sinigaglia M, Corbo MR. Challenges for the production of probiotic fruit juices. Beverages. 2015;1:95–103.

33. Poimenidou SV, Chrysadakou M, Tzakoniati A, Bikouli VC, Nychas GJ, Skandamis PN. Variability of *Listeria monocytogenes* strains in biofilm formation on stainless steel and polystyrene materials and resistance to peracetic acid and quaternary ammonium compounds. Int J Food Microbiol. 2016;237:164–71.

34. Pompermayer DM, Gaylarde CC. The influence of temperature on the adhesion of mixed cultures of *Staphylococcus aureus* and *Escherichia coli* to polypropylene. Food Microbiol. 2000;17:361–5.

35. Pothakos V, Björkroth J. Lactic acid bacteria in food spoilage. In: Vinderola G, Ouwehand A, Salminen S, von Wright A, editors. Lactic acid bacteria: microbiological and functional aspects. Boca Ratón, FL, USA: CRS Press; 2019. p. 303–15.

36. Rendueles O, Ghigo JM. Multi-species biofilms: how to avoid unfriendly neighbors. FEMS Microbiol Rev. 2012;36:972–89.

37. Siroli L, Patrignani F, Serrazanetti DI, Tabanelli G, Montanari C, Tappi S, Rocculi P, Gardini F, Lanciotti R. Efficacy of natural antimicrobials to prolong the shelf-life of minimally processed apples packaged in modified atmosphere. Food Control. 2014;46:403–11.

38. Song H, Lou N, Liu J, Xiang H, Shang D. Label-free quantitative proteomic analysis of the inhibition effect of *Lactobacillus rhamnosus* GG on *Escherichia coli* biofilm formation in co-culture. Proteome Sci. 2021;19:1–14.

39. Taheur FB, Kouidhi B, Fdhila K, Elabed H, Slama RB, Mahdouani K, Bakhrouf A, Chaief K. Anti-bacterial and anti-biofilm activity of probiotic bacteria against oral pathogens. Microb Pathog. 2016;97:213–20.

40. Tarifa MC, Lozano JE, Brugnoni LI. Disinfection efficacy over yeast biofilms of juice processing industries. Food Res Int. 2018;105:473–81.

41. Tarifa MC, Lozano JE, Brugnoni LI. Dual species relations between *Candida tropicalis* isolated from apple juice ultrafiltration membranes, with *Escherichia coli* O157: H7 and *Salmonella* sp. J Appl Microbiol. 2015;118:431–42.

42. Tarrah A, da Silva Duarte V, de Castilhos J, Pakroo S, Junior WJFL, Luchese RH, Fioravante Guerra A, Rossi R, Righetto Ziegler D, Corich V, Giacomini A. Probiotic potential and biofilm inhibitory activity of *Lactobacillus casei* group strains isolated from infant feces. J Funct Foods. 2019;54:489–97.

43. Tejero-Sariñena S, Barlow J, Costabile A, Gibson GR, Rowland I. In vitro evaluation of the antimicrobial activity of a range of probiotics against pathogens: evidence for the effects of organic acids. Anaerobe. 2012;18:530–8.

44. Toushik SH, Kim K, Ashrafudoulla M, Mizan MFR, Roy PK, Nahar S, Kim Y, Ha S-D. Korean kimchi-derived lactic acid bacteria inhibit foodborne pathogenic biofilm growth on seafood and food processing surface materials. Food Control. 2021;129:108276.

45. Trampuz A, Piper KE, Jacobson MJ, Hanssen AD, Unni KK, Osmon DR, Mandrekar JN, Cockerill FR, Steckelberg JM, Greenleaf JF, Patel R. Sonication of removed hip and knee prostheses for diagnosis of infection. NEJM. 2007;357:654–63.

46. Woo J, Ahn J. Probiotic-mediated competition, exclusion and displacement in biofilm formation by food-borne pathogens. Lett Appl Microbiol. 2013;56:307–13.

47. Xu D, Jia R, Li Y, Gu T. Advances in the treatment of problematic industrial biofilms. World J Microbiol Biotechnol. 2017;33:97.

48. Yuan L, Sadiq FA, Wang N, Yang Z, He G. Recent advances in understanding the control of disinfectant-resistant biofilms by hurdle technology in the food industry. Crit Rev Food Sci Nutr. 2021;61:3876–91.