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Research article

Co-cultures of lactic acid bacteria from Brazilian foods as inhibitors of *Listeria monocytogenes* and *Escherichia coli* O157:H7 biofilm formation

Natacha Caballero Gómez [a,*], Julia Manetsberger [a], Nabil Benomar [a], Hikmate Abriouel [a], Bernadette Dora Gombossy de Melo Franco [b]

[a] *Área de Microbiología, Departamento de Ciencias de la Salud, Facultad de Ciencias Experimentales, Universidad de Jaén, Jaén, Spain*
[b] *Food Research Center, University of São Paulo, Faculty of Pharmaceutical Sciences, São Paulo, SP, Brazil*

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ABSTRACT

This work studies co-culture effects of Lactic Acid Bacteria (LAB) such as *Lactobacillus*, *Lactococcus* and *Weissella* strains on the inhibition of biofilm formation in *L. monocytogenes* and *E. coli* O157: H7. The strains were screened for biofilm inhibition as monocultures (single species) as well as in all possible combinations between one *Lactococcus* bacteriocin producer "bac+" (VB69 and VB94) strain and one *Lactobacillus* or *Weissella* non-bacteriocin producer "bac- "strain. Each inoculum contained the lactic acid bacteria (LAB) and pathogenic bacteria in two different ratios (1:10 and 1:100). Results indicated that absolute individual biomass production in biofilm was not enhanced when compared with those of single species biofilms. In L. *monocytogenes* assays (1:00 ratio) a 2-log reduction was observed for the combination of *L. lactis* 69 + *W. viridescens* 113 relative to *L. lactis* 69 alone and a 4-log reduction was observed relative to *W. viridescens* 113. On the other hand, assays against *E. coli* O157: H7 showed that *Lb. helveticus* 352 + *L. lactis* 94 caused a 2-log reduction after 48 h incubation, which dropped to 0.75 log units at 72 h. Our data suggest that the relationship between inoculated microorganisms and the spontaneous food microbiota could be relevant for pathogen inhibition and biofilm development.

1. Introduction

Lactic acid bacteria (LAB) used in food fermentation are able to inhibit or kill food-borne and other pathogenic microorganisms as well as simple spoilage bacteria by producing a variety of antimicrobial agents [1–7]. The competitive microbiota of a product influences the growth of pathogens like *Listeria monocytogenes* [8,9], where for example, previous studies have shown the ability of bacteriocin producing lactic acid bacteria (LAB) to control *Listeria monocytogenes* in packaged sliced cooked meat products (CMP) [10, 11]. However, antagonistic interactions between non-bacteriocin-producing lactic acid bacteria (LAB) and *Listeria monocytogenes* have also been observed [12–17]. In this context, lactic acid bacteria (LAB) that are homofermentative, salt tolerant, psychrotrophic and adapted to meat-based substrates have shown the greatest potential for use as protective cultures for the biopreservation [18]. It is important to note that antimicrobial peptides or proteins produced by lactic acid bacteria (LAB) are small, ribosomally synthesised and possess activity against closely related Gram-positive bacteria. In general, bacteriocin producing strains belong mostly to the

* Corresponding author.
E-mail address: ncgomez@ujaen.es (N.C. Gómez).

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previously named genera *Lactobacillus* and *Lactococcus*, and are classified as Generally Recognised as Safe (GRAS) [19]. The bacteriocins can be of great interest to the food industry, as they are effective against spoilage microorganisms and foodborne pathogens such as *L. monocytogenes* and *Staphylococcus aureus*.

It is important to highlight that the natural habitat of these microorganisms are multispecies communities, in which the community members exhibit complex metabolic interactions. Since microorganisms grow in polymicrobial communities in nature, it is possible that interactions between community members influence their behavior [20–22]. In contrast, biotechnological production processes catalysed by microorganisms are usually carried out with single strains in pure cultures. A number of production processes, however, may be more efficiently catalysed by the concerted action of microbial communities [23,24]. In this regard, it has been shown that co-culture methods can induce the activity of a previously inactive bacteriocin competent strain [25,26] or enhance the production of bacteriocin above a basic level [27,28].

Biopreservation is the application of compounds of "natural" origin, e.g. products resulting from microbial metabolism, to conserve foods. Thus, biopreservation can be broadly defined as the use of safe microorganisms and/or their antimicrobial substances, naturally present or deliberately added to the product, to extend shelf life and improve food safety [29]. Consequently, there is a significant interest in finding new strategies that use natural products to reduce the persistence of pathogens in food-stuffs.

In this regard, the use of lactic acid bacteria (LAB) as protective cultures could be an additional tool to control pathogenic bacteria in matured ready-to-eat meat and dairy products. Lactic acid bacteria (LAB) have a long-standing history as starter or protectives cultures, given their ability to dominate microbial populations of many foods in which they occur naturally. This is due to the production of biologically active compounds such as organic acids, diacetyl, hydrogen peroxide as well as antibacterial peptides and flavour precursors [30]. Therefore, screening natural lactic acid bacteria (LAB) strains for those capable of producing antimicrobial molecules is a promising and proven strategy and a significant number of bacteriostatic or bactericidal compounds produced by lactic acid bacteria (LAB) have been described [31].

In this study, we investigated the *in vitro* activity of specific combinations of *Lactococcus* and *Lactobacillus* or *Weissella* sp. on *L. monocytogenes* and *E. coli* O157: H7 biofilm inhibition. The inhibition of *L. monocytogenes* and *E. coli* O157: H7 biofilm formation was assessed using different combinations of *Lactobacillus*, *Lactococcus* and *Weissella* strains against two different concentrations of *L. monocytogenes* and *E. coli* O157: H7. Also, using crystal violet staining, we assayed the possible interactions like synergism of *Lactococcus* and *Lactobacillus* or *Weissella* sp. on biofilm formation. Here, polystyrene surface was used as a model for studying these interactions. The knowledge obtained in this study may aid in the design of the appropriate mixed starter cultures of lactic acid bacteria (LAB) strains able to improve food safety.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Six lactic acid bacteria (LAB) strains from a lab collection, isolated from food were analysed (Table 1): two bacteriocin-producing *Lactococcus lactis strains* (VB69 and VB94) isolated from Brazilian charqui [32] two non-bacteriocin producing strains *Lactobacillus helveticus* 352 [33] isolated from goat cheese and *Lactobacillus casei* 40 as well as *Weissella viridescens* 113 isolated from ripened cheese (unpublished). The strains were identified by 16S rDNA gene sequencing, according to Cibik et al. [34], using the CEQ2000 XL DNA analysis kit (Beckman Coulter, CA, USA). Lactic acid bacteria (LAB) strains; *Lactobacillus helveticus* 352, *Lactobacillus casei* 40, *Weissella viridescens* and *Lactococcus lactis* VB69 and VB94; were routinely cultured in De Man, Rogosa and Sharpe (MRS) broth (Oxoid, Basingstoke, England) at 30 °C for 18 h. *Escherichia coli* O157:H7 ATCC 35150 and *Listeria monocytogenes* ATTC 7644 were cultured in trypticase soy broth (TSB; Oxoid, Basingstoke, England) at 37 °C for 20 h. The strains were maintained at −80 °C in the appropriate culture broth with 20 % (v/v) glycerol.

2.2. Inoculum preparation

Bacterial cultures were prepared in TSB or MRS, absorbance (A600nm) measured and adjusted to 0.25 ± 0.05 to standardize the number of bacteria (10^7–10^8 CFU/ml). Aliquots (1 ml) of each *Lactococcus* culture were mixed in a sterile test tube with 1 ml of *Lactobacillus* or *Weisella* strains, to prepare the mixture of strains to be tested in biofilm formation experiments. Then, the pathogenic strains *Listeria monocytogenes* ATTC 7644 or *Escherichia coli* O157:H7 ATCC 35150 were added in ratios of 1:100 or 1:10.

Table 1
Bacterial strains used in the study.

Strain	Species	Source	Bacteriocin
VB69	*Lactococcus lactis*	Charqui (Bíscola et al., 2013)	Nisin Z
VB94	*Lactococcus lactis*	Charqui (unpublished)	Nisin Z
40	*Lactobacillus casei*	Rippened cheese (unpublished)	Not producer
352	*Lactobacillus helveticus*	Goat cheese (Silva et al., 2016)	Not producer
113	*Weisella viridescens*	Rippened cheese (unpublished)	Not producer

2.3. Quantification of biofilm formation by mono and dual species consortia

Biofilm formation was quantified according to Borges et al. [35] with some modifications. All possible combinations of two isolates were tested to evaluate potential synergy between two species. Sterile 12-well polystyrene microtiter plates (TPP, Switzer-land) containing 2 ml MRS broth were used; cell numbers (10ˆ7–10ˆ8 CFU/ml) were standardised by adjusting the absorbance (A600 nm) of bacterial suspensions in MRS to 0.25–0.05. Inoculum volumes were 200 μl for monospecific biofilms and 100 μl for each species in bispecific biofilms. Plates were incubated aerobically for 24 h, 48 h, and 72 h at 30 °C. To quantify biofilm formation, the wells were gently washed three times with 2 ml sterile distilled water. The adhering bacteria were fixed with 2 ml methanol (Romyl, Leics, UK) for 15 min, then the microplates were emptied and dried at room temperature. Biofilms were stained with 2 ml of a 2 % (v/v) crystal violet solution added to each well and kept at room temperature for 5 min. Excess stain was removed by placing the plate under gently running tap water. The stain was released from the adherent cells with 2 ml of 33 % (v/v) glacial acetic acid and the optical density (OD) of each well was measured at 595 nm using a plate reader (Microplate reader, Bio-Rad, Hercules; CA, USA).

To assess whether synergistic effects dominated in the individual dual species biofilm, the measured absorbance of the multispecies biofilm (Abs590 MS) was compared to that of the best single species biofilm former present in the relevant combination (Abs590 BS) as follows (Abs590 MS - standard deviation, s.d.): $\geq$ (Abs590 BS s d.) = synergism. Here the following assumptions were made: In the absence of interactions (1) cell density of single species biofilms and multispecies biofilms is equal, so no additional biofilm is formed by multiple species cultures than by single species cultures when similar nutrients are available unless interactions causing synergistic effects occur, and (2) the best biofilm former dominates the biofilm [36].

2.4. Lactic acid bacteria (LAB) co-culture inhibition of L. monocytogenes and E. coli O157:H7 biofilm formation

The strains were screened for the ability to inhibit biofilm formation as single species as well as in all possible combinations between one *Lactococcus* (VB69 and VB94) and one *Lactobacillus* or *Weissella* strain. Each inoculum contained the LAB and pathogenic bacteria in different combinations (see Section 2.2). These combinations (1 % v/v) were inoculated into 2 mL of TSB broth and transferred to 12-well polystyrene microtiter plates (2 mL/well, TPP, Switzerland). Plates were incubated for 24 h, 48 h or 72 h at 30 °C. Half of the broth from the wells was replaced by fresh broth every 24 h. Liquids were gently removed by pipetting after incubation and biofilms were re-suspended by scraping and vigorous agitation. For the count of viable pathogenic microorganisms adhered to the biofilm, three wells for each strain were washed 3 times as described above and scraped. The obtained suspensions were transferred to sterile tubes and vortexed for 30 s. Serial dilutions in 0.85 % saline (w/v) were plated on modified Oxford agar (MOX) for *L. monocytogenes* and MacConkey sorbitol agar (SM) for *E. coli* O157:H7. Plates were incubated at 37 °C for 24–48 h and bacterial counts were determined.

L. monocytogenes and *E. coli* O157:H7 were used as control to monitor the biofilm formation of these strains in ratios of 1:100 and 1:10 in the absence of lactic acid bacteria.

2.5. Statistical analysis

All experiments were repeated three times, with duplicate samples per trial, and results were expressed as average values. Statistical analyses were done using Excel 2016 (Microsoft Corporation, Redmond, WA, United States) to determine averages and standard deviations. Student's *t*-test was also performed. Statistical calculations were based on a confidence level 95 % ($P < 0.05$) which was considered statistically significant.

3. Results

3.1. Biofilm formation by single-species and dual-species consortia

All studied strains were biofilm producers in MRS broth as described by Gómez N. et al. [37]. Moreover, absolute individual biomass production in biofilm was not enhanced when compared with those of single species biofilms, indicating that there is no

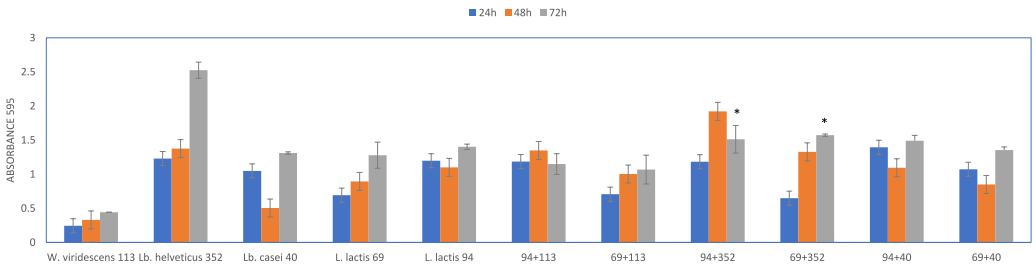

Fig. 1. Biofilm formation of lactic acid bacteria (LAB) co-cultures after 24 h, 48 h and 72 h at 30 °C. A Results are means of triplicates and vertical bars show standard deviations. (*) indicates significant difference ($p < 0.05$) compared to the control (strains alone).

benefit from inclusion in the dual species community (Fig. 1) when cultured at these concentrations. However, for *Lb. helveticus* 352 + *L. lactis* 69 and 94 a decrease of absorbance was observed after 72 h of incubation when compared with *Lb. helveticus* 352 alone, indicating the possibility of an antagonistic interaction.

3.2. Inhibition of *L. monocytogenes* ATTC 7644 biofilm formation

The inhibition was time-dependent and varied according to the strain, combination and proportion of target pathogen (Fig. 2). Firstly, with respect to the two ratios of *L. monocytogenes* tested (1:10 and 1:100), the highest inhibition was observed for the 1:100 ratio, except in the case of the combination of *Lb. casei* 40 + *L. lactis* 69 (Fig. 2D).

When analysing inhibition of individual strains, the highest inhibition relative to the control was obtained for *L. lactis* 69 at the 1:10 ratio, however at the 1:100 ratio the highest log reduction was found for both *L. lactis* 69 and 94 strains (Fig. 2). Regarding the co-cultures between a *Lactococcus* (69 or 94) and a *Lactobacillus* or *Weissella* (40, 352 or 113) strain, the combination of *W. viridescens* 113 + *L. lactis* 69 at the ratio 1:100 stood out, with a 3-2.5-log reduction at 24 h, 48 h and 72 h (Fig. 2B). The combination of *Lb. casei* 40 + *L. lactis* 69 yielded a 2-3-log reduction between 24 h and 72 h for the ratio of 1:10 (Fig. 2C) as well as for the ratio 1:100. On the other hand, a 3-log reduction was observed for the combination of *Lb. casei* 40 + *L. lactis* 69 at 72 h, and a 2-log reduction was detected for *Lb. casei* 40 + *L. lactis* 94 at 24 and 48 h. Finally, in the case of *Lb. helveticus* 352, the combination with *L. lactis* 94 showed the best inhibition results for the 1:100 ratio at 48 h of incubation with a 2.5-log reduction (Fig. 2F).

3.3. Inhibition of *E. coli O157:H7 ATCC 35150* biofilm formation

The inhibition was time dependent and varied according to the strain and proportion of the target pathogen (Fig. 3) similar to the *L. monocytogenes* assays. No reductions were evident in the 1:10 ratio (data not shown). On the other hand, when analysing the 1:100 ratio, the highest inhibition for the individual strains was registered in the case of *L. casei* 40 (Fig. 3A) with a 1-log reduction at 24–48 h and 72 h. In the case of co-cultures, the combination of *L. casei* 40 + *L. lactis* 94 was noteworthy, showing a 2-log reduction at 48 and 72 h (Fig. 3A). *Lb. helveticus* 352 + *L. lactis* 94 showed an inhibition of 2-log at 48 h which dropped to 0.75-log at 72 h of incubation (Fig. 3B), but none was statistically significant.

4. Discussion

In this study, we assessed the ability of *Lactobacillus* or *Weissella* strains co-cultured with bacteriocin producers *Lactococcus lactis* to

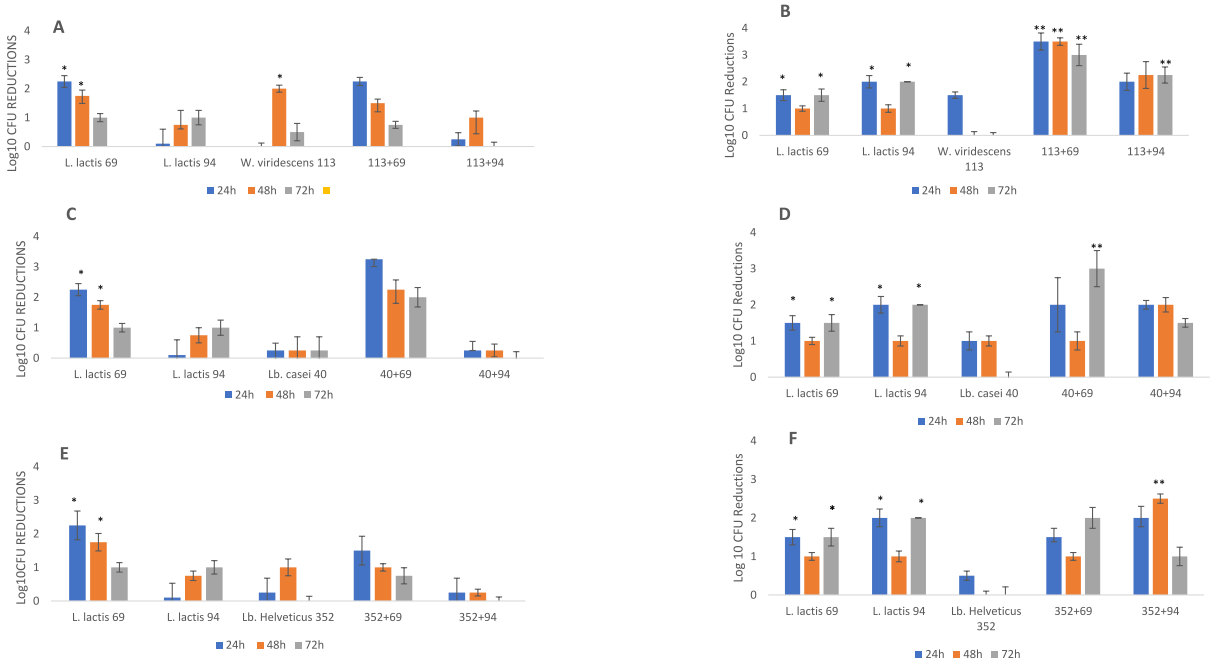

Fig. 2. Log_{10} reduction of *L. monocytogenes* ATTC 7644 biofilms formation (A-C-D) for 1:10 proportion and (B-D-F) 1:100 proportion after 24 h, 48 h and 72 h of incubation.
(*) indicates a statistically significant difference ($p < 0.05$) compared with control and (**) compared with control and two co-cultured strains alone.

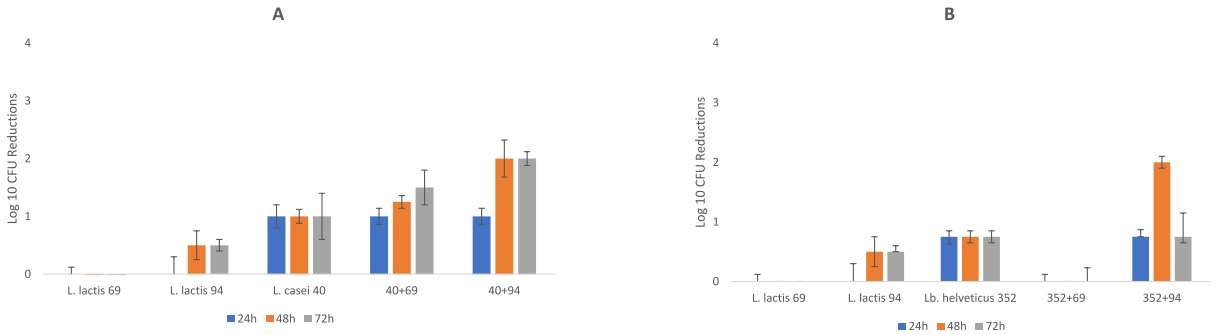

Fig. 3. Log_{10} reduction of *E. coli* O157:H7 ATCC 3515 biofilm formation (A–B) for 1:100 proportion after 24h, 48h and 72 h of incubation. (*) indicates a statistically significant difference ($p < 0.05$) compared with control and (**) compared with control and two co-cultured strains alone.

inhibit *L. monocytogenes* ATTC 7644 and *Escherichia coli* O157:H7 ATCC 35150 biofilm formation *in vitro*. The strains used in this study were characterized as potential probiotics in our previous work and inhibitory action of biofilm formation in pathogens by exclusion was also assayed [37]. In addition, Pacheco da Silva et al. [33] demonstrated a folate and riboflavine production in goat fermented milk by *Lactobacillus helveticus* 352. These previous works highlighted the importance of these strains in the food industry and the present study could lead to the development of new value-added products.

Co-cultured microorganisms have a complex ecological relationships, including synergism, mutualism, mutual antagonism as well as the production of bacteriocins and signalling molecules, which can affect the production of new substances in co-culture systems [38]. In this respect, our results obtained for synergism assays indicated that absolute individual biomass production in biofilms was not enhanced when compared with those of single species biofilms. This indicated that there is no benefit from inclusion in the dual species community for tested concentrations of strains. We can conclude that the increase in pathogenic biofilm inhibition from co-cultures is not due to a major biomass production. That said, for *Lb. helveticus* 352 + *L. lactis* 69 and 94 at 72 h of incubation we observed a decrease of absorbance when compared with *Lb. helveticus* 352 alone. This decrease in biofilm biomass may be due to an antagonistic interaction and could be connected with the promising results obtained for *E. coli* biofilm inhibition by the combination of *Lb. helveticus* 352 with *L. lactis* 94. Some strains of the genus *Lactobacillus* defend their habitat against other Gram-positive bacteria by the secretion of growth inhibiting substances such as nisin or lactain F [39]. In this context, previous works highlighted the impact of starter lactococci cultures on flavour precursor development. They also showed a positive effect of *Lb. helveticus* and the lysis of this strain on enhancing levels of substrate and flavour precursors early during ripening, resulting in early flavour development in Cheddar [40]. In addition, co-cultivation processes can help find new substances of industrial interest, due to the production of a number of secondary metabolites [41].

In our study, the highest rate of co-culture inhibition was reported for 1:100 proportion of *L. monocytogenes* ATTC 7644 and *E. coli* O157:H7 ATCC 3515 cultures. These results indicate major inhibition differences between co-cultures and strains alone with minor pathogen proportions, overlaying the influence of little inoculum variations. Regarding the effect of incubation time, we observed variations in the inhibition depending on incubation time, for example in *W. viridescens* 113 for *L. monocytogenes* inhibition assays (1:10 ratio). For *W. viridescens* 113 + *L. lactis* 69 assays, there was a 2-log reduction of inhibition between 24 h and 72 h, although this reduction of inhibition over time was not observed for experiments using a 1:100 ratio. The same tendency was observer for *Lb. casei* 40 + *L. lactis* 69 and *Lb. helveticus* 352 + 69.

Lactic acid bacteria (LAB) bacteriocins could be applied for the inactivation of Gram-negative pathogens in foods in combination with other hurdles or treatments to induce cell damage and partial disorganization of the outer cell membrane protective layer [42]. Interestingly, our results for *Lb. casei* 40 + 94 against *E. coli* O157:H7 ATCC 3515 (1:100) showed approximately a 1.75-log inhibition increment in biofilm formation compared with bacteriocin producer strain *L. lactis* 94 alone, highlighting the potential use of these co-cultivations as inhibition promoter for Gram-negative bacteria. In addition, it is noteworthy that this inhibition increase was not found for *L. lactis* 69, underlining the differences between strains.

Our data suggests that the interaction between inoculated microorganisms and the natural food microbiota could be relevant for pathogenic inhibition and help prevent biofilm formation. In addition, previous studies have demonstrated variations in the final result of the food fermentation process when co-cultures are present [43–45] which could be another added value of these co-cultures. This will surely be the subject of ongoing and futures studies.

Many studies have shown that *Lactobacillus* can be used in food processing [46,47]. In this regard, Maldonado Barragán et al. [48] showed the induction of plantaricin production by the co-cultivation of *Lactobacillus plantarum* NC8 and *Lactococcus lactis* and the authors hypothesized that a quorum sensing mechanism could be responsible for the induction.

The inhibition results obtained against *L. monocytogenes* are of great interest as this pathogen is a serious concern in RTE meat and matured dairy products. The use of lactic acid bacteria (LAB) combinations as protective cultures could be a promising tool to control *L. monocytogenes*. Although lactic acid bacteria (LAB) strains are present in most matured foods as a natural microbial population, strain selection and evaluation, such as that carried out in our study, is necessary to find more suitable strains with activity against

pathogens.

We further hypothesize that the "natural co-culture" among the inoculated bacteriocin producing strains and the autochthonous lactic acid bacteria (LAB) could lead to their inhibitory effects against pathogenic strains. In this regard, mixed species starter cultures could be previously evaluated in order to increase safety control in foods. The next steps after this study include the evaluation of the selected co-cultures under different maturation/storage conditions and the testing of their anti-listerial activity in food models simulating temperature, water activity and pH conditions as well as evaluation in food matrices. As a result of the proposed isolation and selection methods, lactic acid bacteria (LAB) strains with the ability to produce antimicrobial compounds such as lactic acid and other organic acids, ethanol, diacetyl, carbon dioxide, hydrogen peroxide and bacteriocins will be available.

Further work on the nature of these interactions leading to increased inhibition is currently ongoing. We emphasise that if bacteriocin-producing starter cultures are going to be used in food fermentations, such interactions could be previously tested in order to increase protection against pathogens offering an added value and increasing the safety of the final product. Futures studies are necessary to better understand this interaction. However, given the promising results of biofilm inhibition for *E. coli* O157:H7 and *L. monocytogenes* by competition and exclusion as reported previously Gómez N. et al. [37] as well as the interesting characteristics of these strains (e.g. potential probiotics and vitamins producers), they can be excellent candidates to develop mixed starter cultures to improve food safety and develop new value-added products.

In conclusion, the present study provides evidence for the combination of lactic acid bacteria (LAB) with probiotic potential to produce safer foods, which can possibly be used to develop new efficient starter cultures to inhibit pathogenic biofilms without disrupting the balance of food.

CRediT authorship contribution statement

Natacha Caballero Gómez: Investigation, Funding acquisition, Formal analysis, Conceptualization. **Julia Manetsberger:** Writing – review & editing, Methodology, Investigation. **Nabil Benomar:** Writing – review & editing, Methodology, Investigation. **Hikmate Abriouel:** Writing – review & editing, Investigation, Formal analysis. **Bernadette Dora Gombossy de Melo Franco:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization.

Data availability

Data and materials are available from the corresponding author upon reasonable request.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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