

RESEARCH ARTICLE

Potentially probiotic and bioprotective lactic acid bacteria starter cultures antagonize the *Listeria monocytogenes* adhesion to HT29 colonocyte-like cells

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Abstract

The capability of five lactic acid bacteria (LAB) to counteract the adhesion of *Listeria monocytogenes* to the epithelial intestinal cell line HT29 was studied. The highest adhesion ability to HT29 was achieved by the intestinal strain *Lactobacillus rhamnosus* CTC1679, followed by the meat-derived strains *Lactobacillus sakei* CTC494 and *Enterococcus faecium* CTC8005. Surprisingly, the meat strains showed significantly better adhesion to HT29 than two faecal isolates of *Lactobacillus casei* and even significantly higher than the reference strain *Lb. rhamnosus* GG. Additionally, the anti-listerial, bacteriocin-producer starter culture *Lb. sakei* CTC494 was able to significantly reduce the adhesion of *L. monocytogenes* to HT29 in experiments of exclusion, competition and inhibition. The performance was better than the faecal isolate *Lb. rhamnosus* CTC1679. Our results reinforce the fact that the ability of LAB to interact with a host epithelium model, as well as to antagonize with foodborne pathogens, was a strain-specific characteristic. Additionally, it was underlined that this trait was not dependent on the origin of the bacterium since some food LAB behave better than intestinal ones. Therefore, the search for novel strains in food niches is a suitable approach to found those with potential health benefits. These strains are likely pre-adapted to the food environment, which would make more feasible their inclusion in the formulation of probiotic foods.

Keywords

Lactobacillus, pathogen, adhesion, competition, antagonism

1. Introduction

Lactic acid bacteria (LAB) are one of the main microbial groups involved in spontaneous, as well as controlled, food fermentations. Nowadays the use of LAB strains as cultures to initiate

a fermentative process is a common practice in food industry. The search for novel “functional starters” that can “contribute to the microbial safety or offer one or more organoleptic, technological, nutritional or health advantages” (Leroy and De Vuyst, 2004), is of pivotal relevance for the manufacture of traditional products as well as for the development of new foods (Bourdichon *et al.*, 2012). It is well known that the rapid acidification of the raw (food) material by LAB allows the partial control of food-borne pathogens such as, among others, *Listeria monocytogenes*. Moreover, the use of bacteriocin-producing strains may contribute to reduce the risk of *L. monocytogenes* during dry sausage manufacturing process, thus improving the food safety (Hugas *et al.*, 1995; Työppönen *et al.*, 2003). This pathogen is able to adhere and invade the intestinal epithelium causing a severe disease which is associated with high mortality in immune-compromised populations (EFSA, 2014; Milillo *et al.*, 2012). The ability of LAB strains to exclude *L. monocytogenes* from mucus and intestinal cells has been previously reported (Collado *et al.*, 2007; Gueimonde *et al.*, 2006); such strains could be considered as potential probiotics, i.e., “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2006). Different mechanisms have been proposed to explain the capability of probiotic LAB to counteract the effects of *L. monocytogenes* in the gut; for example, the production of bacteriocins (Corr *et al.*, 2007), the stimulation of the host immune response (Corr *et al.*, 2007) or the modulation of listeria and host transcriptomes by the presence of LAB (Archambaud *et al.*, 2012).

In previous studies, we have showed the high potential as functional starters of several strains belonging to the IRTA culture collection. *Lactobacillus sakei* CTC494, isolated from naturally fermented sausages, is a starter culture producing sakacin K (Aymerich *et al.*, 2000), with proved anti-listerial activity in dry fermented sausages (Hugas *et al.*, 1995). *Enterococcus faecium* CTC8005, a non-virulent and non-aminogenic strain isolated from a meat-processing factory, was able to inhibit the growth of *L. monocytogenes* in low-acid fermented sausages during the whole ripening process, achieving a further reduction in the pathogen counts after a high hydrostatic pressure treatment (Rubio *et al.*, 2013). On the other hand, the strains *Lactobacillus casei/paracasei* CTC1677 and CTC1678, as well as *Lactobacillus rhamnosus* CTC1679, were selected among several LAB isolated from infants’ faeces based on their safety, technological and potential probiotic properties and their capability to lead the fermentation in model sausages (Rubio *et al.*, 2014b) being *Lb. rhamnosus* CTC1679 a suitable starter culture for nutritionally enhanced fermented sausages (Rubio *et al.*, 2014a). In the current work, we want to know whether these five strains could also exert an anti-listerial effect upon gut cells as a step further to characterise their probiotic potential. For that purpose, the human intestinal cellular line HT29 was chosen as *in vitro* model to mimic the colonic epithelium. HT29 monolayers were challenged with different pair combinations of these LAB and *L. monocytogenes* in order to elucidate the ability of different strains to antagonize the adhesion of the pathogen.

2. Materials and methods

Bacterial strains and culture conditions

The strains and culture conditions used in this study are listed in Table 1. As routinely procedure, stocks (with 20% glycerol) of LAB stored at -80°C were surface streaked on de Man, Rogosa and Sharpe (MRS) agar (Biokar Diagnostics, Beauvais, France). *L. monocytogenes* CTC1034 (serovar 4b) was grown on Chromogenic Listeria Agar (CLA, Oxoid Ltd., Basingstoke, Hampshire, England). Plates were incubated for 48 h under different temperatures and O₂ concentration (Table 1). Isolated colonies were picked up to inoculate 10 ml MRS broth, for LAB strains, or 10 ml TSBYE broth (Tryptic Soy Broth Yeast Extract,

Difco Laboratories, Detroit, MI, USA) for *L. monocytogenes*. Cultures were grown overnight under same conditions and were used to inoculate (2% v/v) fresh broths, which were incubated for 24 h. These 24 h-grown cultures were used to prepare bacterial suspensions for the following assays.

Table 1 Bacterial strains and culture conditions used in this study.

Bacteria	Strain ¹	Origin	Culture conditions ²	Reference
<i>Lactobacillus casei</i>	CTC1677	Infants' faeces	MRS, 37°C, anaerobic	Rubio <i>et al.</i> (2014a,b)
<i>Lactobacillus casei</i>	CTC1678	Infants' faeces	MRS, 37°C, anaerobic	Rubio <i>et al.</i> (2014a,b)
<i>Lactobacillus sakei</i>	CTC494	Meat product	MRS, 32°C, anaerobic	Hugas <i>et al.</i> (1995)
<i>Lactobacillus rhamnosus</i>	CTC1679	Infants' faeces	MRS, 37°C, anaerobic	Rubio <i>et al.</i> (2014a,b)
<i>Lactobacillus rhamnosus</i>	LMG18243 (GG)	Culture collection	MRS, 37°C, anaerobic	-
<i>Enterococcus faecium</i>	CTC8005	Meat environment	MRS, 37°C, aerobic	Rubio <i>et al.</i> (2013)
<i>Listeria monocytogenes</i>	CTC1034	Meat product	CLA, 37°C, aerobic	Garriga <i>et al.</i> (2002)

¹ CTC: IRTA culture collection; LMG-BCCM: Belgian co-ordinated collection of microorganisms.

² MRS: de Man Rogosa and Sharpe; CLA: chromogenic listeria agar; anaerobic conditions achieved with Anaerocult A (Merck, Darmstadt, Germany).

Co-aggregation of bacterial strains

The co-aggregation assay was used to detect the aggregation of the pathogen *L. monocytogenes* CTC1034 with the LAB strains and was performed in three independent experiments as previously described (Vandevoorde *et al.*, 1992). Briefly, overnight bacterial cultures were harvested by centrifugation at 2400 ×g for 10 min and washed twice with phosphate buffered saline (PBS) (NaCl, 8g/l; KH₂PO₄, 0.34; K₂HPO₄, 1.21 g/l). The optical density (OD) of the bacterial suspensions was adjusted to 0.60±0.02 at 600 nm with a spectrophotometer (Novaspec Plus, Amersham Biosciences, UK). Equal volumes (2 ml) of LAB and pathogen strains were mixed and shaken for 30 min at 150 rpm and allowing the flocks to settle. Four ml of the bacterial suspensions alone were used as controls. After shaking, the mixtures and the bacterial suspensions alone were incubated for 1 h at room temperature and subsequently the OD at 600 nm was determined. The co-aggregation (%) was expressed according to Handley *et al.* (1987) equation:

$$\text{Co-aggregation (\%)} = \frac{(\text{OD}_{\text{LAB}} + \text{OD}_{\text{Listeria}}) - 2 \times \text{OD}_{\text{mixture}}}{(\text{OD}_{\text{LAB}} + \text{OD}_{\text{Listeria}})} \times 100$$

OD_{LAB}= optical density of LAB strain; OD_{Listeria}= optical density of *L. monocytogenes* CTC1034; OD_{mixture}= optical density of mixed bacterial suspension (LAB + *Listeria*).

Culture conditions of HT29 cell line

The epithelial intestinal cell line HT29 (ECACC 91072201), derived from human colon adenocarcinoma, was purchased from the European Collection of Cell Cultures (Salisbury, UK). The cell line was grown in McCoy's medium supplemented with 10% (v/v) heat-inactivated bovine foetal serum and a mixture of antibiotics (50 µg/ml penicillin, 50 µg/ml streptomycin, 50 µg/ml gentamicin and 1.25 µg/ml amphotericin B). All media and supplements were obtained from Sigma (Sigma Chemical Co., St. Louis, MO). For routine maintenance, the incubations took place at 37°C, 5% CO₂ in a SL Waterjacketed CO₂ Incubator (Sheldon Mfg. Inc., Cornelius, Oregon, USA) following standard procedures. For all experiments, 10⁵ HT29-cells/ml were seeded in 24-well plates and incubated for 11 days until they reached a confluent differentiated state (monolayer, about 10⁷ cells/ml).

Adhesion of single strains to HT29

The ability of the strains, including the reference strain *Lb. rhamnosus* GG, to adhere to HT29 cell line was assayed (Ayeni *et al.*, 2011). For this purpose, 24 h-grown bacterial cultures were harvested by centrifugation (7940 ×g, 10 min), washed twice with phosphate buffered saline (PBS) and resuspended in McCoy's medium without antibiotics at a concentration about 1×10⁸ CFU/ml. HT29 monolayers were washed twice with Dulbecco's PBS buffer (Sigma) to remove the antibiotics and then bacterial suspensions (500 µl) were added at a ratio of about 1:10 (eukaryotic-cell: bacteria). Plates were incubated for 1 h at 37°C, 5% CO₂ in a Heracell® 240 incubator (Thermo Electron LDD GmbH, Langenselbold, Germany). After the incubation period, supernatants were discarded and monolayers were softly washed twice with Dulbecco's PBS buffer to remove the non-attached bacteria. Afterwards, the monolayers were trypsinized to release the eukaryotic cells and bacteria adhered. After appropriate serial dilutions in Ringer solution, counts to determine the number of adhered bacteria were carried out in MRS agar or CLA for LAB or *L. monocytogenes*, respectively. Results were expressed as the percentage of bacteria adhered with respect to the amount of bacteria added (% CFU bacteria adhered/CFU bacteria added). The assay was carried out with two consecutive passages of the cell line and two bacterial duplicates were tests in each one (in two independent wells). Thus each bacterium was tested in four replicates.

Capability of LAB to modify the adhesion of *L. monocytogenes* to HT29

The ability of the LAB strains under study to compete for, and to inhibit or displace the adhesion of *L. monocytogenes* CTC1034 to HT29 monolayers was evaluated following similar procedures to those previously described (Collado *et al.*, 2005). Pair combinations of each LAB with the pathogen were tested in duplicate wells in two replicated plates as follows. To evaluate the ability of the LAB strains to compete with *L. monocytogenes* for adhesion to HT29, both LAB and pathogen strains were simultaneously added in equal volume (250 µl each) of the same bacterial-suspension concentration (about 0.5×10⁸ CFU/ml). After 1 h of incubation at 37°C and 5% CO₂, the percentage of LAB or *L. monocytogenes* adhered was determined by counting as indicated in the previous section.

In the inhibition assay, each LAB strain was first added (500 µl of 10⁸ CFU/ml) alone to wells containing HT29-monolayers and incubated for 1 h at 37°C and 5% CO₂. Thus, the non-adhered cells were removed by washing twice PBS and, then, *L. monocytogenes* CTC1034 was added (500 µl at 10⁸ CFU/ml) over the HT29-monolayer partially colonized with the LAB strain. Afterwards, the plate was incubated for 1 additional hour in the same conditions. For exclusion experiment the order of sequential bacterial addition was changed. First *L. monocytogenes* CTC1034 was added alone, at the same concentration indicated above, and incubated for 1 h with the HT29 cell line. Afterwards, monolayers were washed and then the LAB strains added following 1 h of incubation. At the end of the incubation period (2 h), the

percentage of adhesion for each bacterial strain was determined by counting in the respective selective media.

Changes in the adhesion of *L. monocytogenes* CTC1034 to HT29 due to competition, inhibition or exclusion with each LAB were expressed as percentage of *Listeria* adhered in the presence of the LAB respect to % of *Listeria* adhered alone. Negative values indicate a reduction in the adhesion of listeria promoted by the presence of LAB.

Statistical analysis

The SPSS 19.0 software for Windows (SPSS Inc., Chicago IL, USA) was used to statistically analyse the data by means of independent one-way ANOVA tests and by the post-hoc mean comparison LSD (least significant difference, $p < 0.05$) test. On one hand, the differences in adhesion among the seven strains added independently to HT29 were assessed. On the other hand, for each LAB strain one-way ANOVA tests were performed to compare the adhesion of *L. monocytogenes* to HT29 under the three experimental conditions (competition, inhibition or exclusion) with respect to the adhesion of listeria alone. Differences in *L. monocytogenes* co-aggregation among the five LAB under study were determined as well. In the legend of each figure, the statistical analysis carried out with the corresponding set of data is indicated.

3. Results and discussion

The *in vitro* adhesion to intestinal cell lines is a test often used to evaluate the probiotic potential of novel strains, since the transient colonization of the intestinal epithelium would allow the probiotic to exert its beneficial effect (FAO/WHO, 2006).

The results obtained in our study shown that, in general, all LAB strains presented good adhesion ability to HT29 monolayer (Figure 1). The percentages of adhesion were similar or higher than those of the strain *Lb. rhamnosus* GG, used as reference due to its good adherent properties (Lebeer *et al.*, 2007; Vizoso-Pinto *et al.*, 2007). From the three strains of human origin, only *Lb. rhamnosus* CTC1679 showed significantly higher ($p < 0.05$) adhesion ability than the strain GG. Interestingly, both strains from meat origin (*Lb. sakei* CTC494 and *E. faecium* CTC8005) adhered significantly better to HT29 than *Lb. casei* intestinal strains (CTC1677 and CTC1678) and the reference GG strain. It is well known that the adhesion capability of LAB is a characteristic of strain but not of species (Laparra and Sanz, 2009; Tuomola and Salminen, 1998). Furthermore, closely (isogenic) related strains present different adherence properties to intestinal epithelial cells (Nikolic *et al.*, 2012). This strain-dependent adhesion capability is directly related with the presence of (strain-specific) structural molecules involved in the interaction of bacteria with the environment, such as exopolysaccharides, fimbriae, pili, lipoteichoic acids, secreted proteins, etc. (Lebeer *et al.*, 2010). Our study underlines that the natural niche of these strains did not condition the presence of adhesins able to *in vitro* interact with the host epithelium, since some food LAB attached better to HT29 cells than intestinal-origin ones. The pathogen *L. monocytogenes* CTC1034 also showed a notable adherence (25%) to HT29 monolayers (Figure 1), significantly higher than the reference strain GG (about 6%). It seems that also the ability of *Listeria* to adhere to epithelial cells widely depends on the strain tested (Moroni *et al.*, 2006), which is directly correlated with the surface-molecules fingerprint (Jaradat *et al.*, 2003).

The capability to counteract the effect of pathogens in the gut is one of the desirable characteristics for potential probiotic candidates (FAO/WHO, 2006). The high *in vitro* adhesion of our LAB strains to epithelial intestinal cells prompted us to evaluate their antagonism against the food-borne pathogen *L. monocytogenes*, which in a physiological situation could reach the gut after the ingestion of contaminated foods, invading intestinal

cells, among other virulent traits, and causing disease. In order to *in vitro* evaluate the potential protective effect of the LAB under study, we have chosen the good-adherent listeria strain CTC1034 to perform the experiments of competition, inhibition and exclusion of the pathogen adhesion to HT29 by each strain. The overall view of results depicted in Figure 2 evidenced that the faecal origin, non-bacteriocinogenic *Lb. rhamnosus* CTC1679 and the anti-listerial, meat-derived *Lb. sakei* CTC494 were the most efficient strains exerting antagonism against *L. monocytogenes* adhesion to HT29 monolayers. The remaining strains had not effect or even increased the adhesion of the food-borne pathogen to the cell line; this behaviour has been previously reported for other LAB and enteropathogens (Ayeni *et al.*, 2011; Gueimonde *et al.*, 2006). Therefore, the capability of the LAB to prevent the binding of *L. monocytogenes* to the intestinal epithelium was also a characteristic dependent on the LAB strain (Lavilla-Lerma *et al.*, 2013; Lim and Im, 2012; Nakamura *et al.*, 2012), but not on the food or intestinal origin of the bacteria tested. It is worth noting that *Lb. sakei* CTC494 was the only strain able to significantly ($p<0.05$) reduce the listeria adhesion in the three experimental situations: inhibition, competition and exclusion. Indeed, this was the strain that reduced to a higher extent the adhesion of *L. monocytogenes* CTC1034. Our results showed that the highest adherent strains were also those showing the highest antagonism, i.e., strains CTC1679 and CTC494 (Figure 2). This result reinforced the fact of the existence of a positive correlation between the adhesion properties of potential probiotics and their ability to counteract the adhesion of pathogens such as *L. monocytogenes* (Bouchard *et al.*, 2013).

Regarding putative mechanisms of bacterial antagonism, co-aggregation could be one way of probiotics action preventing the attachment of pathogens to the intestinal surface (Ouwehand *et al.*, 1999; Xu *et al.*, 2009). The probiotic could interact with the pathogen, thus avoiding its binding to the cellular line. In our case, *L. monocytogenes* CTC1034 co-aggregated with *Lb. rhamnosus* CTC1679 which was also able to reduce adhesion of pathogen (Table 2); however, co-aggregation seemed not to be the mechanism of action of *Lb. sakei* CTC494 that was the strain with better anti-listerial performance. Besides, listeria also co-aggregated with *Lb. casei* CTC1677, but this strain was not able to reduce the adhesion of the pathogen to the epithelial cells. These results suggest that capability of some LAB to promote co-aggregation with *Listeria* and to antagonize its adhesion to the cell line model is highly dependent of the strain considered. This characteristic must be related with the presence of specific molecules in the LAB surface able to act either as ligands binding pathogens (Schachtsiek *et al.*, 2004) and/or as adhesins for attachment to the cell line (Walter *et al.*, 2008). Since surface components of LAB are involved in adhesion, co-aggregation and pathogen-adhesion interference, it could be suggested that these phenomena could be interrelated in some specific bacteria. Results obtained in our study support the fact that there are multiple mechanisms by which probiotics exert antagonism to inhibit the adhesion of the pathogens to the intestinal epithelium.

Table 2 Mean values of co-aggregation (%) between each LAB under study and *L. monocytogenes* CTC1034. One way ANOVA was carried out to assess differences among LAB strains (*** $p<0.001$); those having different superscript are statistically different ($p<0.05$) according to the mean comparison test LSD (least significant difference).

	Mean \pm SD ¹
	% co-aggregation <i>L. monocytogenes</i> CTC1034
<i>Lactobacillus casei</i> CTC1677	1.95 \pm 0.05 ^b
<i>Lactobacillus casei</i> CTC1678	-1.37 \pm 1.00 ^a
<i>Lactobacillus sakei</i> CTC494	-0.97 \pm 0.70 ^a
<i>Lactobacillus rhamnosus</i> CTC1679	5.42 \pm 1.57 ^c
<i>Enterococcus faecium</i> CTC8005	-1.31 \pm 0.47 ^a

[†] negative values indicates no co-aggregation

4. Conclusion

Results obtained with *in vitro* models are difficult to extrapolate to the physiological situation in the human GIT. However, the *in vitro* experiments could provide important information regarding the potential of a given strain before to be included in the food carrier and to be tested in human intervention studies. In this work we have shown that *Lb. rhamnosus* CTC1679 and *Lb. sakei* CTC494, both already proved to be suitable starter cultures for fermented sausages, are the best candidates to be used as probiotics to be included in the formulation of fermented foods that could promote health benefits. Next step forward to prove probiotic action will be to test the beneficial effect of these strains in human intervention studies.

Acknowledgements

This work was supported by the Spanish Ministry of Economy and Competiveness (MINECO) through the projects AGL2012-33278 and RTA2009-00045-00-00. R. Rubio is the recipient of a predoctoral fellowship awarded by the INIA (“Instituto Nacional de Investigaciones Agrarias”).

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Figure legends

Figure 1. Adhesion percentage of LAB strains and the pathogen *Listeria monocytogenes* CTC1034 to the human colonocyte-like HT29 monolayer. The strain *Lactobacillus rhamnosus* GG (LMG18243) was used as reference. Differences in adhesion among strains were assessed by means of one-way ANOVA. Bars that do not share a common letter are significantly different according to the mean comparison LSD (less significant difference, $p < 0.05$) test.

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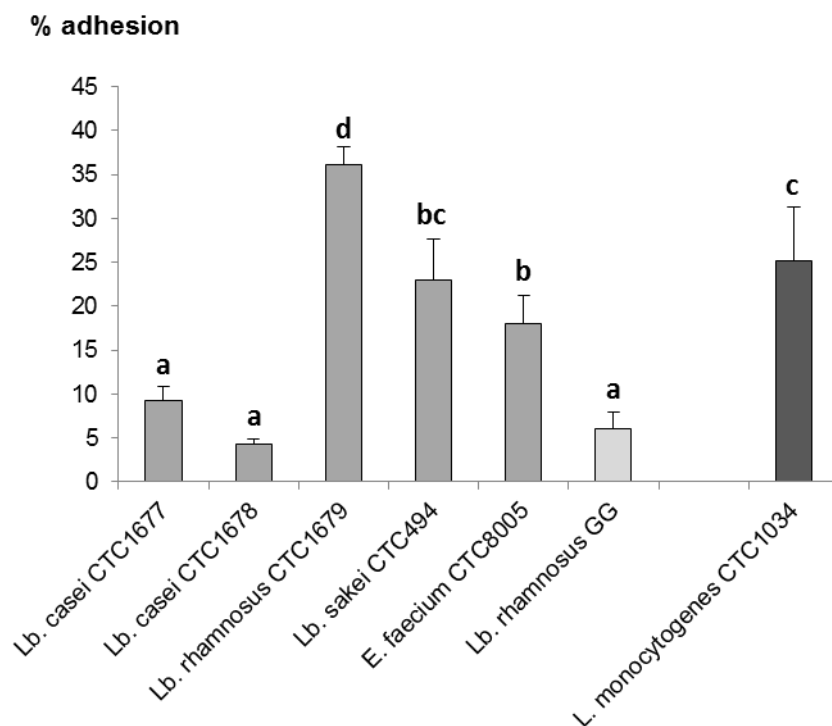


Figure 2. Changes in the adhesion of the pathogen *Listeria monocytogenes* CTC1034 to the human colonocyte-like HT29 monolayer in experiments of competition (black bar), inhibition (light-grey bar) and exclusion (dark-grey bar) with five LAB strains. For each LAB strain, one-way ANOVA test was performed to assess the differences of listeria adhesion among the experimental conditions with respect to the adhesion of the listeria added alone (control). Within the same LAB strain, bars that have an asterisk are significantly different ($p < 0.05$) from the control. The coefficients of variation ($100 \times \text{SD}/\text{mean}$) of the adhesion data ranged between 3% and 29%. Positive values in the Y-axis indicate increases in listeria adhesion to the HT29, with respect to listeria adhered alone (control), whilst negative values indicate decreases of adhesion.

**% Adhesion
Change**

***L. monocytogenes* CTC1034**

