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Inhibition of enteropathogens adhesion to human enterocyte-like HT-29 cells by a dairy strain of *Propionibacterium acidipropionici*

G. Zárate[1*], J.M. Palacios[1], J. Villena[2] and M.E. Zúñiga-Hansen[3]

[1]Centro de Referencia para Lactobacilos, Chacabuco 145, T4000ILC San Miguel de Tucumán, Argentina; [2]Escuela de Medicina, Universidad de Valparaíso, Hontaneda 2653, 234000 Valparaíso, Chile; [3]Escuela de Ingeniería Bioquímica, Pontificia Universidad Católica de Valparaíso (EIB-PUCV), Av. Brasil 2085, Valparaíso, Chile; gzarate@cerela.org.ar

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RESEARCH ARTICLE

Abstract

Adhesion to the host intestinal mucosa is considered relevant for orally delivered probiotics as it prolongs their persistence in the gut and their health promoting effects. Classical propionibacteria are microorganisms of interest due to their role as dairy starters as well as for their functions as probiotics. *Propionibacterium acidipropionici* Q4, is a dairy strain isolated from a Swiss-type cheese made in Argentina that displays probiotic potential. In the present work we assessed the ability of this strain to adhere to the human enterocyte-like HT-29 cell line and to counteract the adhesion of two common human enteropathogens, such as *Escherichia coli* C3 and *Salmonella* Enteritidis 90/390. The results were compared with those obtained with the well-known probiotic *Lactobacillus rhamnosus* GG. *P. acidipropionici* Q4 showed a high adhesion capacity, even higher than the reference strain *L. rhamnosus* GG (42.3±4.4% and 36.2±2.3%, respectively), whereas adhesion of enteropathogens was significantly lower (25.2±2.2% for *E. coli* and 21.0±3.4% for *S.* Enteritidis). Propionibacteria as well as lactobacilli were able to inhibit by exclusion and competition the adherence of *E. coli* C3 and *S.* Enteritidis 90/390 whereas only *L. rhamnosus* GG displaced *S.* Enteritidis from HT-29 intestinal cells. Inhibition of pathogens by propionibacteria was not exerted by antimicrobials or coaggregation but was mainly due to exclusion by cell surface components, such as proteins and carbohydrates. The relevance of cell surface proteins (CSP) for preventing pathogens infection was confirmed by their concentration dependent effect observed for both pathogens: 100 µg/ml of CSP inhibited *E. coli* attachment almost as untreated propionibacteria, whereas it partially inhibited the attachment of *S.* Enteritidis. Results suggest that *P. acidipropionici* Q4 could be considered for the development of propionibacteria containing functional foods helpful in counteracting enteropathogen infection.

Keywords*:* propionibacteria, probiotics, enteropathogens, adhesion

1. Introduction

Probiotics are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2002). In this respect, several properties have been proposed as selection criteria for microorganisms intended for orally delivered probiotics, such as acid and bile resistance, attachment to the intestinal mucosa, antagonism against pathogens and technological properties (Collado *et al.,* 2010; Tripathi and Giri, 2014). Among them, survival to the adverse conditions of the gastrointestinal tract and persistence in high number in the gut to allow them time enough to exert their beneficial effects seem to be crucial. It is thought that to produce effects in the host, probiotics have to be consumed at a level that varies from 10^6 to 10^9 cfu/ml and remain in the intestine in spite of the normal washout by peristalsis of the gut (Tripathi and Giri, 2014). Then, microorganisms with a high growth rate and/or the ability to adhere to mucosal surfaces will have a prolonged persistence in the body of the host and would be preferred for inclusion in functional foods. In fact, adhesion of probiotic microorganisms to the intestinal mucosa is considered the first step in colonisation and has been shown relevant for many probiotic health

effects, such as microbiota and immune system modulation (Collado *et al.,* 2010; Ouwehand and Salminen, 2003).

On the other hand, adhesion to epithelial cells by pathogenic bacteria is considered an important prerequisite for the onset of intestinal infections. Attachment of enteropathogens to cell surfaces may lead to cell damage, internalisation, disturbances of regulatory cell mechanisms, intracellular proliferation and colonisation. In the case of invasive bacteria, they cross the epithelial membrane, proliferate and promote cell death and exfoliation (Ribet and Cossart, 2015).

Adhesion of both probiotics and pathogens is mediated by adhesins of the bacterial surface and complementary mucosal receptors of the host cell (Lebeer *et al.,* 2010). Thus, when selecting microorganisms for probiotic purposes, the adherent strains are preferred in order to form a biological barrier against pathogens colonisation and thereby prevent the infections. This 'anti-infective' mechanism is based mainly on the inhibition of pathogenic bacteria adhesion by competitive exclusion, i.e. blocking of receptors with specific adhesin analogues or by steric hindrance (Collado *et al.,* 2010; Ofek *et al.,* 2003).

Different studies have shown that adhesive probiotic bacteria have the ability to prevent the attachment of pathogens and/or stimulate their removal from intestinal (Garriga *et al.,* 2014; Gueimonde *et al.,* 2006; Lee *et al.,* 2003; Tareb *et al.,* 2013) and urogenital cells (Osset *et al.,* 2001; Zárate and Nader-Macías, 2006). Most of these studies have focused on the use of lactobacilli and bifidobacteria as protective microorganisms since they are common inhabitants of the gastrointestinal and urogenital tracts and belong to genera most frequently used as probiotics (Collado *et al.,* 2010; Tripathi and Giri, 2014). However, in the last decades other microorganisms like propionibacteria have gained prominence as probiotics for human and animal health (Zárate and Pérez Chaia, 2015).

Classical propionibacteria are microorganisms of interest due to their technological properties as dairy starters, biological producers of propionic acid and other important biomolecules (vitamins B and K, conjugated linoleic acid, extracellular polysaccharide (EPS), trehalose, bacteriocins) as well as for their probiotic properties (Cousin *et al.,* 2011; Zárate and Pérez Chaia, 2015). Both *in vitro* and *in vivo* studies have demonstrated that propionibacteria are able to modulate in a favourable manner gut physiology, microbiota composition and immunity (Zárate and Pérez Chaia, 2015). In this sense, *Propionibacterium freudenreichii* and *Propionibacterium acidipropionici*, have been included in the list of agents recommended for Qualified Presumption of Safety (QPS) by the European Food Safety Authority (Leuschner *et al.,* 2010).

P. acidipropionici Q4, is a dairy strain isolated from a Swiss-type cheese made in Argentina, that displays probiotic potential as it possesses high b-galactosidase activity (Zárate and Pérez Chaia, 2012a; Zarate *et al.,* 2003), modifies colonic fermentation of lactose (Pérez Chaia and Zárate, 2005), and decreases intestinal b-glucuronidase activity (Pérez Chaia *et al.,* 1999). It also resists the adverse conditions of the gastrointestinal tract (Zárate *et al.,* 2000) and adheres to intestinal epithelial cells of mice (Zárate *et al.,* 2002a).

In the present study, we assessed *in vitro* the ability of *P. acidipropionici* Q4 to adhere to the human HT-29 cell line taken as an intestinal mucosa model and to inhibit the adherence of two human enteropathogens, *Escherichia coli* C3 and *Salmonella* Enteritidis 90/390 as a step further to characterise its probiotic potential.

2. Materials and methods

Microorganisms and growth conditions

P. acidipropionici Q4 was isolated in our laboratory from an Argentinean Swiss-type cheese and characterised for probiotic properties in CERELA, Argentina (Pérez Chaia and Zárate, 2005; Pérez Chaia *et al.,* 1999; Zárate *et al.,* 2000, 2002a, 2003). *Lactobacillus rhamnosus* GG was used as probiotic reference strain and was provided by EIB-PUCV, Chile. *E. coli* C3, a clinical isolate obtained from faeces of a child diagnosed with enteropathogenic diarrhoea was obtained from the Institute of Microbiology 'Luis Verna' of the University of Tucumán, whereas *Salmonella enterica* serovar Enteritidis 90/390 was provided by the Culture Collection of Estación Experimental Agropecuaria Balcarce, INTA, Argentina.

Before experimental use, the strains stored at -20 °C in 10% (w/v) reconstituted skim milk containing 5 g/l yeast extract were reactivated by three successively transfers every 24 h in their respective growth media. Propionibacteria and lactobacilli were grown at 37 °C under static conditions (without agitation) in De Man, Rogosa and Sharpe (MRS) broth (Oxoid, Basingstoke, UK), whereas enteropathogens were cultured on brain heart infusion broth (Oxoid) with shaking at 100 rpm.

Intestinal epithelial cell line

The human enterocyte-like HT-29 cell line was used as *in vitro* intestinal model for adhesion assays. This human colon adenocarcinoma derived cell-line was provided by the Medicine School of Valparaiso University, Chile (originally obtained from the American Type Culture Collection, Rockville, MD, USA). Cells were routinely grown in D-MEM/F12 medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated (30 min at 56 °C) foetal bovine serum and a mixture of antibiotics

(50 U/ml penicillin, 50 µg/ml streptomycin, and 1.25 µg/ml amphotericin B) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. For all experiments, monolayers of HT-29 cells were prepared in 24-well tissue-culture plates by inoculating 2×10^4 viable cells per well in 1 ml culture medium. Cells were incubated until they reached a confluent differentiated state (about 10^7 cells/ml) with a change of culture medium every 2 days.

Adhesion assays

Adhesion of each strain (lactobacilli, propionibacteria and enterobacteria) individually to intestinal cells was assessed first. With this aim, microorganisms at stationary phase of growth (overnight cultures of lactobacilli and pathogens and 48 h-grown cultures of propionibacteria) were centrifuged (5,000×*g*, 10 min, 4 °C) and the supernatants were discarded. The pellets were washed twice with phosphate buffered saline (PBS) and resuspended in D-MEM/F12 low glucose, without antibiotics, at a concentration of about 1×10^8 cfu/ml. HT-29 monolayers were washed three times with Dulbecco's PBS buffer to remove the antibiotics and then 1 ml of bacterial suspensions were added to each well (a ratio of about 1 eukaryotic cell : 10 bacteria). Plates were incubated for 1 h at 37 °C under 5% CO_2 atmosphere. After that, culture medium was removed and monolayers were washed three times with sterile saline solution to remove free and slightly bound bacterial cells. Afterwards, the monolayers were detached and disrupted with 1 ml of a solution containing 0.5% (v/v) Triton X-100 and 0.25% (v/v) trypsin in PBS. The resultant lysates were transferred to tubes containing 9 ml of PBS, serially diluted and plated on MRS and MacConkey agars (BD Difco, Franklin Lakes, NJ, USA) for enumeration of probiotics and enterobacteria, respectively. MRS plates were incubated at 37 °C in anaerobiosis during 72 h and 120 h for growth of lactobacilli and propionibacteria, respectively. MacConkey was incubated at 37 °C for 24 h under aerobic conditions. Results were expressed as adhesion percentage and calculated as follows:

$$\% \text{ Adhesion} = \frac{\text{cfu/ml after adhesion}}{\text{cfu/ml before adhesion}} \times 100 \quad (1)$$

Inhibition of adhesion assays

The ability of propionibacteria and lactobacilli to inhibit adhesion of *E. coli* C3 and *S.* Enteritidis 90/390 to HT-29 monolayers was evaluated following procedures previously described. Three types of assays were performed: blockage by exclusion, by competition or by displacement (Zárate and Nader-Macías, 2006). For the exclusion test, suspensions of potential probiotics (500 µl of 10^8 cfu/ml) were added to HT-29 intestinal cells and incubated for 60 min at 37 °C under 5% CO_2-95% air atmosphere, then pathogens suspensions (500 µl of 10^8 cfu/ml) were added and the mixtures were incubated for another 60 min under the same conditions. For the competition assay both potential probiotics and pathogens were simultaneously added in equal volume (500 µl each) of the same bacterial suspension concentration (about 1×10^8 cfu/ml) to HT-29 monolayers and were incubated at 37 °C for 120 min.

For the displacement test, intestinal cells and pathogens were mixed first and incubated together for 60 min; potential probiotics were added later and incubation was continued for further 60 min. After the incubation periods, monolayers were washed to remove unbound bacteria, disrupted and plated on selective culture media for enumeration of adhered bacteria as described in the previous section. Changes in the adhesion of pathogens to HT-29 cells due to exclusion, competition, or displacement with each probiotic strain were expressed as % Inhibition according to the following formula:

$$\% \text{ Inhibition} = \frac{\text{Adhesion of pathogen alone} - \text{adhesion of pathogen with potential probiotics}}{\text{Adhesion of pathogen alone}} \times 100 \quad (2)$$

Factors involved in adhesion inhibition

The antimicrobial activity of *P. acidipropionici* Q4 on both enteropathogens was determined by assessing the viability of *S.* Enteritidis 90/390 and *E. coli* C3: (1) after co-incubation of each pathogen with propionibacteria in cell culture medium (D-MEM/F12 low glucose without antibiotics) during 120 min; (2) after suspension for 120 min in the propionibacteria spent medium. Co-aggregation of propionibacteria with pathogens was assessed as described by Tareb *et al.* (2013). Equal volumes (2 ml) of tested bacterial cells (propionibacteria and pathogens) were mixed and incubated at room temperature for 120 min. The absorbance (600 nm) was determined for the mixture and for the bacterial suspensions alone. The percentage of coaggregation was determined as:

$$\% \text{ Coaggregation} = \frac{\left(\frac{(A_{\text{propionibacteria}} + A_{\text{pathogen}})}{2}\right) - A_{\text{mix}}}{(A_{\text{propionibacteria}} + A_{\text{pathogen}})/2} \times 100 \quad (3)$$

In other assays, propionibacterial cells were subjected to different chemical and enzymatic treatments in order to remove cell surface components prior to assays for inhibition of pathogens adherence. One ml aliquots of the propionibacterial suspension were centrifuged (5,000×*g*, 10 min) and resuspended in 1 ml of the following treatment solutions (Zárate *et al.,* 2002b): (1) sodium metaperiodate (0.05 M) in 0.1 M phosphate-citrate buffer, pH 4.6, 60 min; (2) lipase (2 mg/ml) in 0.1 M acetate buffer, pH 5.0, 60 min; (3) 5M LiCl, 15 min, followed by pronase E/Trypsin (1 mg/ml each one) in 0.05 M phosphate buffer, pH 7.8, 45 min; (4) 30% trichloroacetic acid, 180 min; (5) heat treatment (PBS at 80 °C), 30 min. Suspensions were incubated with

gentle agitation (150 rpm) at 37 °C with exception of (4) that was incubated at 4 °C. After treatments, cells were washed, resuspended to their original volume in PBS (10^8 bacteria/ml), and used immediately for exclusion and competition assays with enteropathogens. Propionibacteria suspended in PBS were used as control. Viability of propionibacteria after treatments was checked by plating on MRS media. Adhesion of heat-killed propionibacteria was determined by counting remaining bacteria in wells under the microscope according to Li *et al.* (2008). In brief, HT-29 cells were seeded on a coverslip in the bottom of a 6-well tissue culture plate. After adhesion assay and removal of unbound bacteria by repeated washings with PBS, cells were fixed with methanol, Gram stained and dried overnight at 37 °C. Coverslips were mounted inverted on albumin coated glass slides and the attached bacteria were counted at 1000× with oil immersion objective in 20 randomly chosen microscopic fields. The results were expressed as the ratio of heat-killed adherent bacteria to viable adherent bacteria (determined by the same method).

Inhibition of pathogens adhesion by cell surface protein extract from propionibacteria

Cell surface protein extracts (CSP) were prepared using the method described by Ren *et al.* (2012). Briefly, propionibacteria were grown in 100 ml of MRS broth for 48 h, harvested by centrifugation and washed twice in PBS at pH 7.4. Cells were incubated for 1 h at 37 °C in 10 ml of 5 M LiCl and pronase/E-Trypsin (1 mg/ml each one) in 0.05 M phosphate buffer, pH 7.8. After that the suspensions were centrifuged to remove bacterial biomass and the supernatants were filtered through a 0.2 mm filter, dialysed overnight against 10 mM Tris-HCl (pH 7.4) at 4 °C, lyophilised, and resuspended in PBS at a concentration of 10 and 100 µg/ml of protein according to Bradford procedure (corresponding to approx. 8.7 and 9.7 log cfu/ml, respectively). The cell surface of propionibacteria before and after treatment were observed by transmission electron microscopy as described in Zárate *et al.* (2002b). In brief, samples were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, washed three times with 0.1 M cacodylate buffer at 10 min-intervals and postfixed in 2% osmium tetroxide in 0.1 M cacodylate buffer for 2 h. Cells were washed three more times with the same buffer, dehydrated with ascending concentrations of ethanol and acetone and embedded in SPRLV Spurr kit (Sigma, St. Louis, MO, USA). Thin sections were cut with an ultramicrotome to a thickness of 70 nm, stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (EM 109, Zeiss, Oberkochen, Germany).

The role of CSP extract on pathogens inhibiton was determined in exclusion assays as described in ' Inhibition of adhesion assays' using: (1) untreated propionibacteria; (2) LiCl/proteases treated propionibacteria; (3) surface extract.

Statistical analysis

The results are expressed as the means ± standard deviation of six replicates (the assays were carried out with two consecutive passages of the cell line and two bacterial duplicates in three different wells each one). Significant differences between mean values were determined by Tukey's test after analysis of variance (one way ANOVA) with Minitab release 12 statistical software for Windows (Minitab Inc., State College, PA, USA). A $P<0.05$ was considered to be statistically significant.

3. Results

Probiotic lactobacilli, propionibacteria and enteropathogens used in the present study were able to adhere to different degrees to HT-29 cells used as intestinal model. High adhesion levels were observed for both *P. acidipropionici* Q4 and *L. rhamnosus* GG (42.3±4.4% and 36.2±2.3% respectively, $P>0.05$), whereas adhesion of enteropathogens was significantly lower ($P<0.05$) with means of 25.2±2.2% for *E. coli* C3 and 21.0±3.4% for *S.* Enteritidis 90/390 (Figure 1).

Figure 2 shows the effect of propionibacteria and lactobacilli on the attachment of *E. coli* 90/390 and *S.* Enteritidis to HT-29 cells under the conditions of exclusion, competition and displacement.

Propionibacteria as well as lactobacilli interfered to different extents with the adherence of the enteropathogens tested. The inhibition of the adhesion of pathogens by probiotic strains was dependent on the probiotic strain and the pathogen assayed. For instance, *P. acidipropionici* Q4

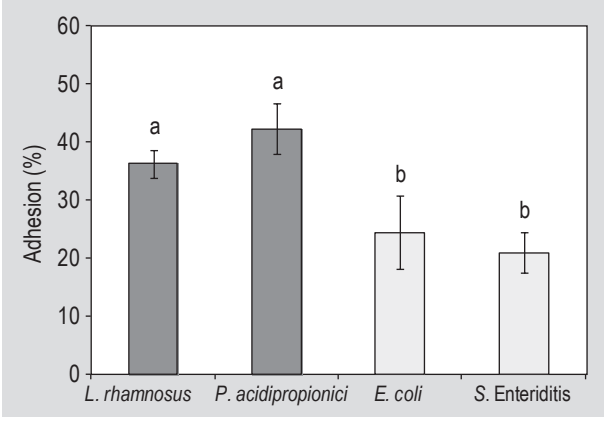

Figure 1. Percentage of adhesion (bacteria adhered with respect to the amount of bacteria added) of *Lactobacillus rhamnosus* GG, *Propionibacterium acidipropionici* Q4 and enteropathogens *Escherichia coli* C3 and *Salmonella* Enteriditis 90/390 to HT-29 intestinal cell line. Results presented are the mean ± standard deviation of cells bound for six wells from two independent experiments. Bars that do not share the same letter are significantly different ($P<0.05$).

blocked by exclusion 29.85±5.87% of *E. coli* C3 adherence and 82.36±2.07% of *S.* Enteritidis 90/390 adherence whereas *L. rhamnosus* GG inhibited these pathogens by the same mechanism in 86.49±2.14% and 87.25±2.95% respectively.

Both lactobacilli and propionibacteria were able to exclude, and compete with both pathogens for adhesion to HT-29 cells (Figure 2). However, only *L. rhamnosus* GG was able to displace *S. enteritidis* from intestinal cells in a significant manner (53.41±4.02%). *P. acidipropionici* Q4 was able to inhibit *S.* Enteritidis adhesion to HT-29 cells by exclusion and that of *E. coli* by competition and displacement to the same level of the recognised probiotic *L. rhamnosus* GG. However, it was less efficient than this probiotic strain to exclude *E. coli* and to compete and displace *S.* Enteritidis from HT-29 cells ($P<0.05$) (Figure 2).

To gain understanding on the mechanisms by which propionibacteria inhibit the adhesion of enteropathogens to intestinal epithelial cells, antimicrobial activity of propionibacteria, coaggregation with pathogens and adhesion inhibition, after removal of their surface structures, were assayed. No decrease of enteropathogen counts was observed after their coincubation with propionibacteria or suspension in propionibacteria spent media (data not shown). Low coaggregation was observed after 2 h between propionibacteria and *E. coli* (5.3±1.1%) or *S.* Enteritidis (4.9±1.6%).

Since propionibacteria exerted no significant displacement of enteropathogens (Figure 2), only exclusion and competition assays were performed with propionibacteria deprived of surface structures. Removal of surface proteins, carbohydrates and teichoic acids with LiCl-proteases, metaperiodate and TCA, respectively, decreased adhesion of *P. acidipropionici* Q4 to intestinal cells ($P<0.05$), whereas treatments with lipase used to remove lipids did not modify it in a significant manner ($P>0.05$) (Figure 3). In the same way, inactivation of the cells by a heat treatment slightly improved adhesion of *P. acidipropionici* Q4, although the increase did not reach statistical significance ($P>0.05$).

When propionibacteria deprived of surface proteins and carbohydrates (LiCl-proteases and metaperiodate treated cells) were used to inhibit pathogens adhesion, an increase of pathogens counts, i.e. less inhibition, was observed in relation to the inhibition exerted by intact propionibacteria ($P<0.05$) (Figure 4, Table 1). On the contrary, lipase and heat-treated propionibacteria were as efficient as untreated cells for inhibiting enteropathogens adhesion (Table 1). Adhesion inhibitions were not abolished in any case by removal of one type of surface structures or by killing propionibacteria suggesting that more than a single structure is involved in inhibition and that viability is not relevant for blocking pathogens adhesion to intestinal cells (Figure 4). In the case of *Salmonella*, a significant decrease ($P<0.05$) of inhibition was observed when propionibacteria were deprived of surface proteins and carbohydrates, whereas *E. coli* inhibition could be ascribed mainly to proteinaceous factors (Table 1).

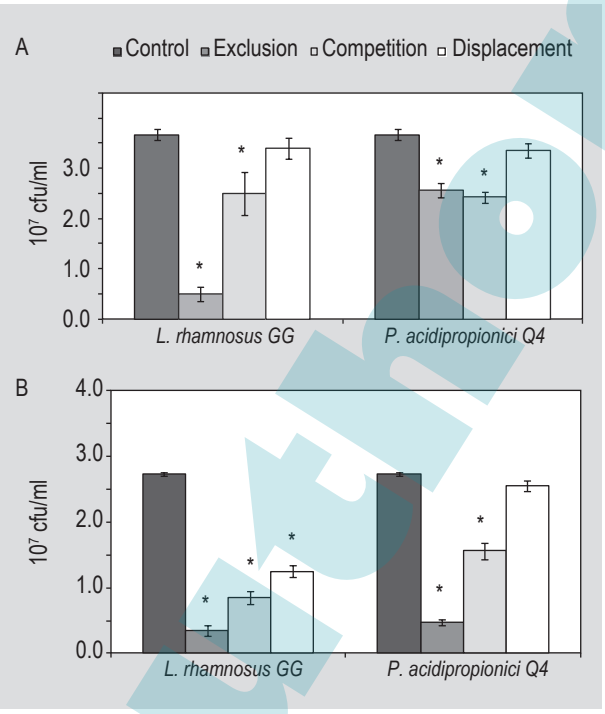

Figure 2. Inhibition of adhesion to intestinal epithelial cells of two enteropathogens: (A) *Escherichia coli* C3 and (B) *Salmonella* Enteritidis 90/390 by *Lactobacillus rhamnosus* GG and *Propionibacterium acidipropionici* Q4 under the conditions of exclusion, competition, and displacement. Bars with an asterisk are significantly different ($P<0.05$) from their respective control.

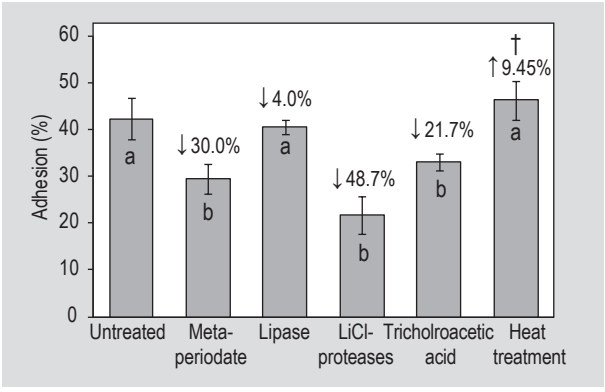

Figure 3. Adhesion of intact *P. acidipropionici* Q4 or bacterial cells deprived of some surface components to HT-29 cell line. Percentages above each bar shows the change in adhesion as compared to untreated cells taken as control. † Adhesion of heat killed bacteria was determined by counting Gram stained bacteria compared to adhesion of viable bacteria determined by the same method.

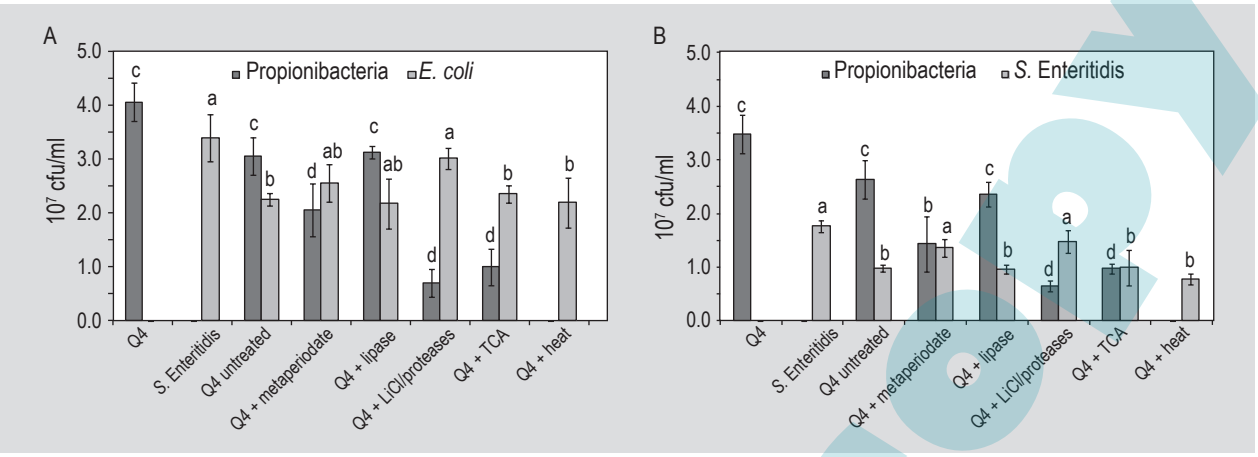

Figure 4. Adhesion of enteropathogens and propionibacteria to HT-29 cells under competition with intact *Propionibacterium acidipropionici* Q4 (Q4) or cells deprived of surface components. Adhesion was determined by a plate count method and the adherence of each microorganism alone was taken as control. Each bar represents mean ± standard deviation of the three data from two assays. (A) *Escherichia coli* plus *P. acidipropionici* Q4 (B) *Salmonella* Enteritidis plus *P. acidipropionici* Q4. Each microorganism was compared with its own control (pathogen: letters a, b; propionibacteria: c and d, the same letter means *P*>0.05).

Table 1. Effect of intact *Propionibacterium acidipropionici* Q4 or cells deprived of some surface components on adhesion of *Escherichia coli* and *Salmonella* Enteritidis to intestinal epithelial cells under the conditions of exclusion and competition.

		Inhibition (%)[1]			
		E. coli C3		*S.* Enteritidis 90/390	
Treatment of propionibacteria	Surface structure	Exclusion	Competition	Exclusion	Competition
Untreated	none	29.85±5.87[a]	33.94±4.79[a]	82.36±2.07[a]	42.88±3.97[a]
Metaperiodate	carbohydrates	23.25±3.34[a]	27.80±2.77[a]	46.40±5.67[b]	23.16±3.78[b]
Lipase	lipids	30.67±1.46[a]	30.86±4.33[a]	80.22±4.44[a]	45.76±4.56[a]
LiCl-proteases	S-layer/proteins	14.80±4.04[b]	13.44±3.66[b]	25.88±3.68[b]	16.38±6.32[b]
TCA	teichoic acids	26.80±2.68[a]	29.52±3.87[a]	77.62±1.46[a]	43.62±2.42[a]
Heat killed	-	31.60±6.42[a]	34.80±5.02[a]	79.54±5.32[a]	44.66±5.04[a]

[1] Inhibitions are expressed as percentages (mean ± standard deviation) compared to adhesion inhibition of pathogens without probiotics presence (control values taken as 0%). Values with different letter in the same column are significantly different (*P*<0.05).

Since removal of surface proteins showed a greater effect on adhesion of propionibacteria (Figures 3 and 5) and also in the inhibition of adherence of both pathogens, their role was verified by an assay that compared the effect of intact propionibacteria, those deprived of surface proteins and the extract containing them on pathogens exclusion.

As shown in Figure 6, the ability of propionibacteria to inhibit pathogens adherence was strikingly reduced after the surface proteins were removed by LiCl/proteases (as confirmed by transmission electron photomicrographs). However, different levels of exclusion were observed when two concentrations of CSP were preincubated with intestinal cells prior to pathogens addition confirming their importance for preventing pathogens infection. Involvement of surface proteins was also confirmed by their concentration dependent effect observed for both pathogens. In the case of *E. coli*, 100 µg/ml of CSP almost conserved the inhibitory effect of untreated propionibacteria whereas it only exerted a partial inhibitory effect on the attachment of *S.* Enteritidis.

4. Discussion

Ability to adhere to the surface of epithelial cells is a key factor for both probiotics and intestinal pathogens as it is related with health benefits and disease, respectively. Adhesion is also considered one of the main selection

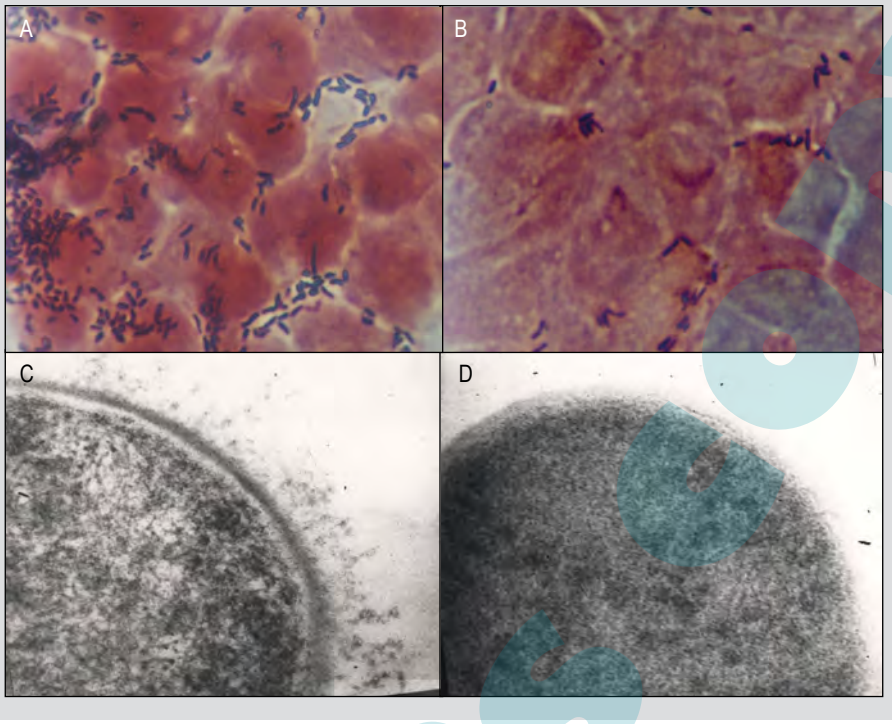

Figure 5. Adherence and cell surface appeareance of untreated (A and C) and LiCl/proteases treated (B and D) *Propionibacterium acidipropionici* Q4 to intestinal cells observed by light microscopy after Gram staining (1000× magnification) and transmission electron microscopy (230,000× magnification).

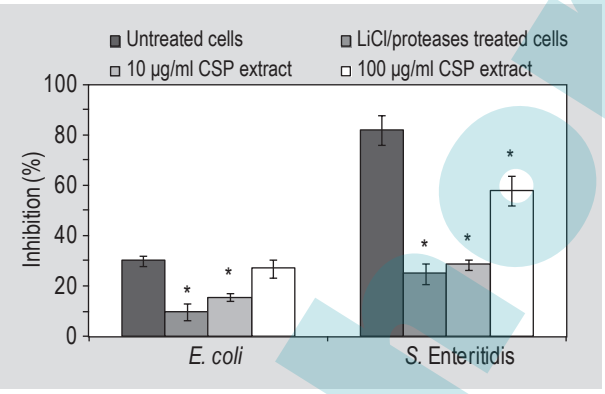

Figure 6. Inhibition of *Escherichia coli* C3 and *Salmonella* Enteriditis adherence by exclusion with untreated cells, LiCl/proteases treated cells and cell surface protein (CSF) extracts of *Propionibacterium acidipropionici* Q4. Bars with asterisks are significantly different (*P*<0.05) from untreated cells.

criteria for probiotic microorganisms (FAO/WHO, 2002; Ouwehand and Salminen, 2003). Different methods are commonly used as *in vitro* tools to assess adhesive properties of probiotic bacteria and to evaluate their interactions with pathogens at the intestinal mucosa. (Laparra and Sanz, 2009; Ouwehand and Salminen, 2003).

In the present study the human enterocyte like HT-29 cell line was chosen as the *in vitro* model to mimic the intestinal epithelium for assessing the ability of the potential probiotic strain *P. acidipropionici* Q4 to adhere and antagonise the adhesion of two common enteropathogens. The well-known *L. rhamnosus* GG was used as reference strain. As expected and previously shown by other authors, LGG presented good adhesion ability to HT-29 monolayer (Dhanani and Bagchi, 2013; Zivkovic *et al.,* 2015). However, the dairy *P. acidipropionici* Q4 showed a percentage of adhesion higher than that of the reference *L. rhamnosus* GG. In this respect it has been proposed that adhesion capability of probiotics (as well as their antagonism against pathogens) is a strain-dependent feature related with the presence of strain-specific structural molecules (exopolysaccharides, fimbriae, pili, teichoic and lipoteichoic acids, surface proteins, etc.) involved in the interaction of bacteria with their surroundings (Lebeer *et al.,* 2010; Sengupta *et al.,* 2013). Additionally, it has been underlined that this trait is not dependent on the origin of the bacterium, since some food bacteria behave better than intestinal ones (Garriga *et al.,* 2014).

Previous studies have shown that this propionibacterial strain has the ability to adhere to mouse intestinal cells both *in vitro* and *in vivo* (Zárate *et al.,* 2002a). Since propionibacteria grow slowly in natural environments and culture media, adhesion ability becomes an important property in the selection of strains for probiotic purposes. Dairy propionibacteria have demonstrated to adhere

to immobilised mucus (Thiel *et al.,* 2004; Zárate *et al.,* 2002b); to isolated intestinal cells (Zárate *et al.,* 2002a), to human intestinal cell lines (Huang and Adams, 2003; Lehto and Salminen, 1997; Ranadheera *et al.,* 2012), and *in vivo* to intestinal cells as was assessed by counting the adhering propionibacteria on the mucosa by plate count methods (Huang and Adams, 2003; Pérez Chaia and Zárate, 2005; Zárate and Pérez Chaia, 2012b). Interactions of propionibacteria with the host gut mucosa were also suggested by the analysis of the genome and surface proteome of *P. freudenreichii* that revealed the presence of a high number of surface proteins involved in adhesion and also present in other probiotic bacteria (Falentin *et al.,* 2010; Le Maréchal *et al.,* 2015).

Both *Salmonella* Enteritidis and Enteropathogenic *E. coli* (EPEC) tested in this work were also able to adhere to HT-29 cells. *Salmonella* infection is one of the primary causes of gastroenteritis in humans; and although symptoms are usually mild they can be distressing for the patient and even fatal in a small percentage of cases. Similarly, EPEC is known to cause chronic, watery diarrhoea in humans, primarily young children and infants. Central to these enterobacteria-mediated diseases is their colonisation of the intestinal epithelium.

One of the primary benefits associated with probiotics is the exclusion of pathogenic bacteria. The most frequently cited reasons for this activity include the production of antimicrobial substances, such as bacteriocins and organic acids, the stimulation of the host immune response, coaggregation and interference of adhesion to the intestinal walls (Collado *et al.,* 2010; Lebeer *et al.,* 2010; Ouwehand and Salminen, 2003).

Inhibition of pathogens by antimicrobials produced by propionibacteria as well as immunomodulation exerted by this genus has been described by several studies (for a review see Zárate and Pérez Chaia, 2015). However, *P. acidipropionici* Q4 used in the present research showed no inhibition of enteropathogens by antimicrobials or significant co-aggregation with them.

Adhesive cultures of potential probiotics have demonstrated inhibitory activity against intestinal attachment of many common enteropathogens, e.g. *E. coli*, *Salmonella* spp., *Listeria monocytogenes, Clostridium difficile, Staphylococcus aureus, Enterobacter sakazakii* and *Campylobacter jejuni* among others (Garriga *et al.,* 2014; Gueimonde *et al.,* 2006; Lee *et al.,* 2003; Ren *et al.,* 2012; Tareb *et al.,* 2013; Zivkovic *et al.,* 2015). Therefore, we investigated the ability of potential probiotic propionibacteria to block the adherence of two frequent enteropathogens by three possible mechanisms: exclusion by adhered probiotics, competition for receptor sites and displacement of adhered pathogens. Exclusion supposes that probiotics recognise first some common receptors or specific ones sterically hindering pathogens attachment. In this test, potential probiotics were allowed to adhere to HT-29 cells first and each of the pathogens was added later. Results showed that both propionibacteria and lactobacilli adhered on the surface of intestinal cells were able to exclude *E. coli* and *S.* Enteritidis. Competition is determined by the affinity of adhesins on the respective bacterial surfaces for the specific receptors that they are competing for; or their relative positions in the case of steric hindrance. Competition between pathogens and probiotics for adhesion on the surface of HT-29 cells was also observed. In this case, adherence of *S.* Enteritidis was higher inhibited by *L. rhamnosus* GG than *P. acidipropionici* Q4. Finally, the ability to displace pathogens from intestinal epithelial cells indicates that affinity of probiotics for the specific receptors is higher than that of the pathogenic strain tested. In the present study, when pathogens were allowed to adhere to HT-29 cells first and potential probiotics were then added, some degree of displacement of *S.* Enteritidis and *E. coli* was observed. However, only *L. rhamnosus* GG was able to reduce the adherence of *Salmonella* to intestinal cells in a significant level. Inhibition of adhesion of *E. coli* and *Salmonella* species by *L. rhamnosus* GG on mucus and intestinal cells was reported previously (Lee *et al.,* 2003).

Regarding dairy propionibacteria, their ability to inhibit exogenous and opportunistic pathogens by competitive exclusion and production of antimicrobials has been demonstrated for strains extensively studied, such as *P. freudenreichii* strain JS (Collado *et al.,* 2007; Myllyluoma *et al.,* 2008) and *P. acidipropionici* CRL 1198 (Lorenzo-Pisarello *et al.,* 2010; Pérez Chaia and Zárate, 2005; Pérez Chaia *et al.,* 1999; Zárate and Pérez Chaia, 2012b).

As mentioned, different bacterial cell surface macromolecules are involved in the interaction of bacteria with the gastrointestinal mucosa being in many cases similar molecules for both commensals and pathogens (Lebeer *et al.,* 2010). Bacterial genome studies (Azcarate-Peril and Klaenhammer, 2010) have also suggested that probiotics use mechanisms similar to those of pathogens to survive and colonise the gut. Then, competitive exclusion of pathogens from their binding sites on the cells, by competition or blockage, has been described for many probiotics, such as lactic acid bacteria and bifidobacteria (Gueimonde *et al.,* 2006). The effect has been ascribed mainly to surface proteins (S-layer, sortase-dependent proteins, lipoproteins) with specific binding ability to intestinal cells and extracellular matrix components, and to a lesser extent to non-proteinaceous adhesins (wall teichoic acids, lipoteichoic acids and EPS) (Sengupta *et al.,* 2013). In this respect, it has been shown that surface layer proteins from different *Lactobacillus* strains inhibit the adhesion of *E. coli* and *S. typhimurium* to different cell lines (Chen *et al.,* 2007; Johnson-Henry *et al.,* 2007; Ren *et al.,* 2012). In the same way, carbohydrates and teichoic acids of probiotics

have been used to protect cells against pathogens, like *Candida albicans, S. aureus* or *L. monocytogenes* (Livins'ka *et al.,* 2013; Zivkovic *et al.,* 2015). In the present study both surface proteins and carbohydrates seem to be involved in the inhibition of *Salmonella,* whereas proteins were mainly responsible for inhibition of *E. coli.* In propionibacteria cell surface proteins (InlA, LspA, SlpE, SlpA and SlpB), probably involved in interactions with the host, have been reported recently for *P. freudenreichi* ITG P20 (Le Marechal *et al.,* 2015) whereas different cell surface factors were involved in adherence of *P. acidipropionici* CRL 1198 to intestinal epithelial cells (Zárate *et al.,* 2002b). Although teichoic acids seemed to act as adhesins in propionibacteria, these molecules were not involved in competition with pathogens for receptors in the host cell. Since different adhesins could be involved in host interactions, a multi-component system acting on different types of adhesins should be more effective at blocking pathogenic bacterial adhesion to enterocytes, as proposed by Ofek *et al.* (2003).

Regarding viability, inactivation with heat was observed to affect the adhesion in many strains (Ouwehand *et al.,* 2000; Tareb *et al.,* 2013). In the current study, propionibacteria exhibited a tendency to increase adhesion following heat treatment. The mechanism involved has not yet been elucidated but it may be due to physicochemical rearrangement of the surface structures that are involved in the interaction with the intestinal mucosa. Similar observations were made by Ouwehand *et al.* (2000) who reported an increased adhesion of *P. freudenreichii* subsp. *shermanii* JS after heat inactivation.

It is generally considered that probiotics should be viable in order to exert beneficial health effects. However, the capacity of the heat-killed probiotic cells to retain their adhesion and exclusion abilities could be considered a selective advantage, enabling these strains to remain functional in harsh conditions, such as technological processing and gastrointestinal transit. Results of the present study suggest that *P. acidipropionici* Q4 could contribute to prevent colonisation of the gastrointestinal tract by relevant pathogens, such as *E. coli* and *Salmonella* through barrier and interference mechanisms (mainly exclusion and competition). Since propionibacteria was not able to displace pathogens it must be emphasised that the administration protocol has to be considered in order to exert the proposed beneficial effect. To our knowledge this is the first report of competitive exclusion of enteropathogens by the surface adhesins of *P. acidipropionici*.

As conclusion, data suggest that *P. acidipropionici* Q4 have potential as anti-infective agent in the gastrointestinal tract and could be considered for inclusion in functional foods helpful in preventing intestinal infections. Further *in vivo* studies designed to test propionibacteria capacity to manage gastrointestinal infections caused by these pathogens are currently underway using an animal model.

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