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Selected *Lactobacillus* strains isolated from sugary and milk kefir reduce *Salmonella* infection of epithelial cells *in vitro*

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RESEARCH ARTICLE

Abstract

The isolation of potentially probiotic strains and the subsequent study of their properties are very important steps to gain insight in the health benefits ascribed to sugary and milk kefir. The aim of the present study was to characterise fifteen *Lactobacillus* strains isolated from these beverages by determining some surface properties and their ability to antagonise enterocyte cell damage after *Salmonella* infection *in vitro*. *Lactobacillus* surface properties were determined by hydrophobicity, autoaggregation, and coaggregation assays with *Salmonella*. In addition, lactobacilli adhesion to Caco-2/TC-7 cells and the effect on *Salmonella* invasion were evaluated. Finally, the disassembly of F-actin cytoskeleton on intestinal epithelial cells was assayed *in vitro* when *Salmonella* infection was performed in the presence of selected *Lactobacillus* strains. Ten out of the 15 strains showed a high adhesion capacity to Caco-2/TC-7 cells. Most of the strains were hydrophilic and non-autoaggregating. Strains isolated from sugary kefir were non-coaggregating with *Salmonella,* while strains *Lactobacillus paracasei* CIDCA 83120, 83121, 83123, 83124, 8339, 83102 isolated from milk kefir were able to coaggregate after 1 h. *L. paracasei* CIDCA 8339 and *Lactobacillus kefiri* CIDCA 83102 were able to diminish *Salmonella* invasion to the enterocytes. An antagonistic effect on cytoskeleton disruption elicited by the pathogen was also demonstrated. Our results suggest that both strains isolated from milk kefir could be considered as appropriate probiotic candidates.

Keywords: coaggregation, autoaggregation, hydrophobicity, adhesion, invasion

1. Introduction

The growing demand for healthier foods is stimulating the innovation and development of new products in the food industry worldwide. Among foods with alleged health claims, the ones containing probiotic strains have received considerable attention. Probiotic strains of lactic acid bacteria (LAB) with the potential to alleviate gastrointestinal diseases, prevent infection of pathogenic bacteria, and degrade carcinogens in the gut, have been used in the manufacture of functional food and pharmaceutical products (Granato *et al.,* 2010).

Although many probiotic microorganisms were originally isolated from the human intestine, other sources such as artisanal fermented products also exist. Kefir is a fermented beverage traditionally consumed for their health promoting properties, and is produced by fermentative activity of 'kefir grains' containing a wide variety of mainly LAB, acetic acid bacteria and yeasts. Milk kefir grains are commonly grown in cow´s milk and the fermented product is called 'kefir' (Garrote *et al.,* 2001). Water (or sugary) kefir grains are grown in a solution of raw sugar and water, and the fermented product is known as 'sugary kefir', 'water kefir' or 'aquakefir' (Gulitz *et al.,* 2011). The

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origin of water kefir remains unclear. It has been postulated that the polysaccharide grains originate from the leaves of the Opuntia cactus fig plant (Gulitz *et al.,* 2011). Pidoux *et al.* (1990) called them 'sugary kefir grains' in order to differentiate them from the grains used for milk fermentation.

Genera present both in milk and water kefir grains include *Lactobacillus, Leuconostoc, Lactococcus,* and *Acetobacter*, and the yeast genera include *Kluyveromyces, Candida, Saccharomyces, Torulaspora, Kazachstania, Lachancea,* and *Yarrowia,* while species present in both grains are different (Ferreira-Zanirati *et al.,* 2015; Garrote *et al.,* 2001; Gulitz *et al.,* 2011; Marsh *et al.,* 2013; Miguel *et al.,* 2010). Although there are many studies about the probiotic properties of *Lactobacillus* isolated from milk kefir (Chen *et al.,* 2012; Golowczyc *et al.,* 2007, 2008; Kakisu *et al.,* 2012; Powell *et al.,* 2007), little is known on the probiotic properties of *Lactobacillus* isolated from sugary kefir (Ferreira-Zanirati *et al.,* 2015).

Among the most important intestinal pathogens, *Salmonella* is one of the most extensively studied and characterised. This pathogen is able to interact with the intestinal epithelium brush border, conducting to the formation of typical surface appendages or membrane 'ruffling' (Chen *et al.,* 1996; Ginocchio *et al.,* 1994). *Salmonella* can then invade intestinal cells by vacuole formation containing *Salmonella* cells (Steele-Mortimer *et al.,* 2002), causing significant alterations to the F-actin cytoskeleton network (Finlay *et al.,* 1991; Guignot *et al.,* 2004; Méresse *et al.,* 2001). Probiotic strains isolated from different origins have been found to inhibit the infection of intestinal epithelial cells by *Salmonella* sp. (Burkholder and Bhunia, 2009; Golowczyc *et al.,* 2007; Lee *et al.,* 2003; Schierack *et al.,* 2011).

Both the presence of kefir microorganisms as well as their metabolites (such as lactic acid, carbon dioxide, acetaldehyde, acetoin, alcohol, specific flavour compounds, etc.) contributes to the beneficial effects of this product. Although many probiotic strains are already available for commercial use (Collado *et al.*, 2007), isolation and characterisation of new strains from natural sources with a long tradition of health promotion are still desirable. Kefir grains and fermented products represent a rich source of LAB biodiversity, which contributes to the health-promoting characteristics traditionally ascribed to this beverage. Moreover, isolation of potentially probiotic strains and subsequent study of their properties are important steps to gain insight in the health benefits ascribed to sugary and milk kefir.

As part of a larger investigation in our working group about the selection and characterisation of new probiotic candidates, the aim of the present study was to characterise fifteen *Lactobacillus* strains isolated from milk and sugary kefir. We studied both some surface properties as the interaction of selected *Lactobacillus* isolates with intestinal epithelial cells and their effect on *Salmonella* infection.

2. Materials and methods

Origin of strains, identification and growth conditions

Milk kefir strains were previously isolated and identified (Hamet *et al.,* 2013). Sugary kefir strains were isolated and identified in Ghent University from a Belgian commercial aquakefir product. Strains were identified through a polyphasic approach combining $(GTG)_5$-PCR profiling and partial 16S rRNA and/or phenylalanyl-tRNA synthase (*PheS*) gene sequencing as previously described (Hamet *et al.,* 2013). The *Lactobacillus* strains used in the present study encompassed the species *L. paracasei*, *L. diolivorans, L. harbinensis, L. hilgardii* and *L. kefiri*, and are listed in Table 1.

Stock cultures were stored in 120 g/l non-fat milk solids at -80 °C. Working cultures were cultivated in De Man, Rogosa, Sharpe broth (MRS, Difco, Detroit, MI, USA) for 48 h at 30 °C under static conditions. *Salmonella enterica* serovar Enteritidis CIDCA 101 (Golowczyc *et al.,* 2007) was used for adhesion/invasion experiments. It was grown in nutrient broth (Biokar Diagnostics, Beauvais, France, ref no. BK003HA) for 18 h at 37 °C.

Caco-2/TC-7 cell cultures

Caco-2/TC-7 cells, which model the mature enterocytes of the large intestine, were used. Cells were routinely grown in Dulbecco modified Eagle's minimal essential medium (DMEM) (Gibco BRL Life Technologies Rockville, MD, USA), supplemented with 15% (v/v) heat-inactivated (30 min, 60 °C) foetal calf serum (GEN SA., Buenos Aires, Argentina), 1% (w/v) non-essential amino acids (Gibco BRL), penicillin (12 IU/ml), streptomycin (12 µg/ml), gentamicin (50 µg/ml) and fungizone (1.25 µg/ml) (Parafarm, Saporiti SACIFIA, Buenos Aires, Argentina). Cells were seeded at a concentration of 2.5×10^5 cells per well in 24-well tissue culture plates (GBO Corning, Corning, NY, USA) and incubated at 37 °C in a 5% CO_2 – 95% air atmosphere. Culture medium was changed every two days. Caco-2/TC-7 cells were used at post-confluence after 7 days of culture (differentiated cells) at passages between 23 and 26.

Hydrophobicity assays for microbial adhesion to hydrocarbons

The protocol was followed according to Pérez *et al.* (1998). Briefly, two ml of *Lactobacillus* cell suspension (OD=1 equivalent to 2×10^8 cfu/ml) in phosphate buffered saline (PBS) (130 mM sodium chloride, 10 mM sodium

Table 1. *Lactobacillus* and *Salmonella* strains used in the present study, origin and references.

Strain[1]	Species	Origin	Reference
R-40086[a]	*L. paracasei*	Sugary kefir	This paper
R-40003[a]	*L. paracasei*	Sugary kefir	This paper
R-40122[a]	*L. paracasei*	Sugary kefir	This paper
R-39998[a]	*L. paracasei*	Sugary kefir	This paper
R-40006[a]	*L. paracasei*	Sugary kefir	This paper
R-40076[a]	*L. paracasei*	Sugary kefir	This paper
R-40110[a]	*L. diolivorans*	Sugary kefir	This paper
R-40024[a]	*L. harbinensis*	Sugary kefir	This paper
R-40124[a]	*L. hilgardii*	Sugary kefir	This paper
CIDCA 83120[b] / R-45465[a]	*L. paracasei*	Milk kefir	Hamet *et al.*, 2013
CIDCA 83121[b] / R-45466[a]	*L. paracasei*	Milk kefir	Hamet *et al.*, 2013
CIDCA 83123[b] / R-45468[a]	*L. paracasei*	Milk kefir	Hamet *et al.*, 2013
CIDCA 83124[b] / R-45469[a]	*L. paracasei*	Milk kefir	Hamet *et al.*, 2013
CIDCA 8339[b] / R-45463[a]	*L. paracasei*	Milk kefir	Hamet *et al.*, 2013
CIDCA 83102[b] / R-45464[a]	*L. kefir*	Milk kefir	Hamet *et al.*, 2013
CIDCA 101[b]	*Salmonella enterica* serovar Enteritidis	Clinical isolate	Golowczyc *et al.*, 2007

[1] [a] = LMG code; [b] = CIDCA code.

phosphate, pH 7.2) were mixed with 0.4 ml of xylene by vortexing for 120 s. The phases were allowed to separate by decantation (5-10 min), and the A_{600} was measured by using a spectrophotometer (Metrolab 330, Buenos Aires, Argentina). The decrease in the absorbance of the aqueous phase was taken as a measure of the cell surface hydrophobicity (H%), as calculated with the formula: $H\% = (A_o - A)/A_o \times 100$, where A_o and A are the absorbance before and after extraction with xylene, respectively.

Autoaggregation assays

Lactobacillus cells were harvested at stationary phase, collected by centrifugation (10,000×*g* for 10 min), washed twice, and resuspended in PBS. In all experiments, *Lactobacillus* suspensions were standardised to OD_{600}=1.0 (2×10^8 cfu/ml). Optical density was measured in a spectrophotometer (Metrolab 330) at regular intervals without disturbing the microbial suspension to determine the kinetics of sedimentation. The autoaggregation coefficient (AC) was calculated at 1 h according to Golowczyc *et al.* (2007) as: $AC = [(OD_t - OD_i)/OD_i]\times 100$ where OD_i is the initial optical density at 600 nm of the microbial suspension and OD_t is the optical density at time *t*.

Coaggregation assays

Lactobacillus cell suspensions were obtained as described above. *Salmonella* cells were harvested in stationary phase by centrifugation for 4 min at 5,000×*g* and resuspended in PBS. 1 ml of *Lactobacillus* suspension (2×10^8 cfu/ml) and 1 ml of *Salmonella* suspension (2×10^8 cfu/ml) were mixed in glass test tubes and incubated at 37 °C. Optical density was measured at zero time and after 1 and 20 h without shaking the glass tube. Coaggregation coefficient (CC) was calculated at time *t* according to Golowczyc *et al.* (2007) as: $CC = [(OD_t - OD_i)/OD_i] \times 100$ where OD_i is the initial optical density at 600 nm of the microbial suspension and OD_t is the optical density at time *t*. Coaggregation coefficient (CC) was calculated as stated before but subtracting the corresponding aggregation coefficient (AC) of each strain obtained as previously explained.

Lactobacillus adhesion to Caco-2/TC-7 cells

The adhesion assay was carried out according to Golowczyc *et al.* (2007). Briefly, Caco-2/TC-7 monolayers at post-confluence were washed twice with sterile PBS buffer (pH 7.2) and were incubated with 0.5 ml of each *Lactobacillus* suspension (1×10^8 cfu/well) and 0.5 ml of DMEM for 1 h at 37 °C in a 5% CO_2-95% air atmosphere. Then, monolayers were washed and lysed by adding 1 ml of sterile distilled water. To determine the number of viable lactobacilli that adhered to Caco-2/TC-7 cells, appropriate dilutions in 0.1% (w/v) tryptone solution in water were plated on MRS and colony counts were performed after 48 h incubation. Experiments were carried out in triplicate on three consecutive cell passages.

Galley proof

Salmonella association and invasion assays

Assays were carried out according to Golowczyc *et al.* (2007). Briefly, to evaluate *Salmonella* association, 0.5 ml of *Salmonella* suspension (1×10^8 cfu/well) and 0.5 ml of DMEM were added to each well and incubated 1 h at 37 °C in a 5% CO_2 – 95% air atmosphere. Then, monolayers were washed three times and lysed by adding sterile water. Appropriate dilutions in 0.1% tryptone were plated and colony counts were performed in order to determine the number of associated (adhering plus invading) viable cells.

Two different types of experiments were performed to evaluate *Salmonella* invasion: (1) Caco-2/TC-7 cells were first preincubated with 0.5 ml *Lactobacillus* suspension (1×10^8 cfu/well) for 1 h at 37 °C as described above and washed; then, 0.5 ml of *Salmonella* suspension (1×10^8 cfu/well) and 0.5 ml of DMEM was added to each well. Control cells were preincubated 1 h with PBS (without *Lactobacillus* suspension) (2) *Lactobacillus* (1×10^8 cfu/well) and *Salmonella* (1×10^8 cfu/well) suspensions were mixed and coincubated in PBS (pH 7.2) for 1 h at 37 °C; then, 0.5 ml of the mixture and 0.5 ml of DMEM was added to each well. *Salmonella* control was coincubated with PBS (without *Lactobacillus* suspension). At the end of coincubation *Salmonella* count was performed and no evidence of a decrease in viable cells during this period was observed. With the aim of counting only internalised bacteria, Caco-2/TC-7 cells were washed twice with PBS and incubated for 1 h at 37 °C with 0.5 ml of gentamicin 100 µg/ml (Sigma-Aldrich, St. Louis, MO, USA) in PBS. Next, the monolayers were washed twice with PBS, lysed by adding sterile water for 1 h and colony counts were performed on nutrient agar (Biokar Diagnostics, ref no. BK021HA). Experiments were carried out in triplicate on three consecutive cell passages.

F-actin cytoskeleton labelling

The F-actin cytoskeleton was labelled with fluorescein-labelled phalloidin (Coconier *et al.*, 2000). Briefly, monolayers of Caco-2/TC-7 cells prepared on glass coverslips were incubated for 1 h with 0.5 ml selected *Lactobacillus* strains (1×10^8 cfu/well). Next, monolayers were washed twice with PBS and were subsequently infected with *Salmonella* (1×10^8 cfu/well) for 1 h. After infection, cells were washed with PBS and fixed with 3% (w/v) paraformaldehyde in PBS for 15 min. Samples were then treated with 50 mM NH_4Cl for 10 min to block aldehyde functions, permeabilised for 4 min with Triton X-100, and incubated for 45 min with 1 µg/ml fluorescein-labelled phalloidin (Sigma-Aldrich). Then, the coverslips were mounted in 50% (v/v) glycerol-0.1% (v/v) sodium azide in PBS. Samples were examined by an inverted microscope system (Nikon TI-Eclipse) coupled to NIS-Elements imaging software (Nikon Instruments Inc., Tokyo, Japan).

Data analysis

Results were expressed as means ± standard deviation of at least three separate duplicate experiments. Statistical analysis was performed with the GraphPad Prism version 5.0 for Windows (San Diego, CA, USA). A simple analysis of variance (ANOVA) to determine differences was used. The significant differences between the mean values of each experiment were determined by the Tukey test (confidence interval 95%).

3. Results

Adhesion to Caco-2/TC7 cells, hydrophobicity and autoaggregation of *Lactobacillus* isolates

The studied strains showed variable adhesion values to intestinal cells. *L. paracasei* strains R-40076 and R-40003 isolated from sugary kefir showed adhesion values lower than 2 $\log_{10}$ cfu/well, while the rest of the studied strains showed adhesion values higher than 4 $\log_{10}$ cfu/well (Figure 1).

Most *Lactobacillus* strains were hydrophilic when tested with xylene as they exhibited hydrophobicity percentages <5%. Only *L. diolivorans* R-40110 isolated from sugary kefir, showed a hydrophobicity percentage of 18.1±2.5%.

Autoaggregation ability of lactobacilli depended on the strains. *L. diolivorans* R-40110, *L. paracasei* strains R-40003 and R-40076, showed autoaggregation percentages >10%, while other strains autoaggregated less than 9% (Table 2).

Interaction of lactobacilli with *Salmonella* and implications on pathogen invasion

Coaggregation of total lactobacilli with *Salmonella* was evaluated at 1 and 20 h. Strains isolated from milk kefir showed the highest coaggregation percentages after 1 h (between 27 and 39%) compared to strains isolated from sugary kefir. After 20 h, *Lactobacillus* showed different percentages of coaggregation with *Salmonella*. *L. paracasei* strains R-40086 and R-39998, *L. diolivorans* R-40110 and *L. hilgardii* R-40124 showed coaggregation values lower than 2.2%, while *L. paracasei* CIDCA 8339 and *L. kefiri* CIDCA 83102 showed the highest coaggregation coefficient (62.6 and 72.5%, respectively) (Table 2).

Ability to inhibit pathogen invasion to enterocytes was performed only for strains with high adhesion ability to Caco-2/TC-7 cells. *L. paracasei* strains R-40086, R-39998, CIDCA 8339, CIDCA 83120, CIDCA 83121, CIDCA 83123, and CIDCA 83124, *L. diolivorans* R-40110, *L. hilgardii* R-40124 and *L. kefiri* CIDCA 83102 were coincubated with *Salmonella*, after that, the monolayers Caco-2/TC-7 were infected and the number of internalised *Salmonella* was

Uncorrected Proof

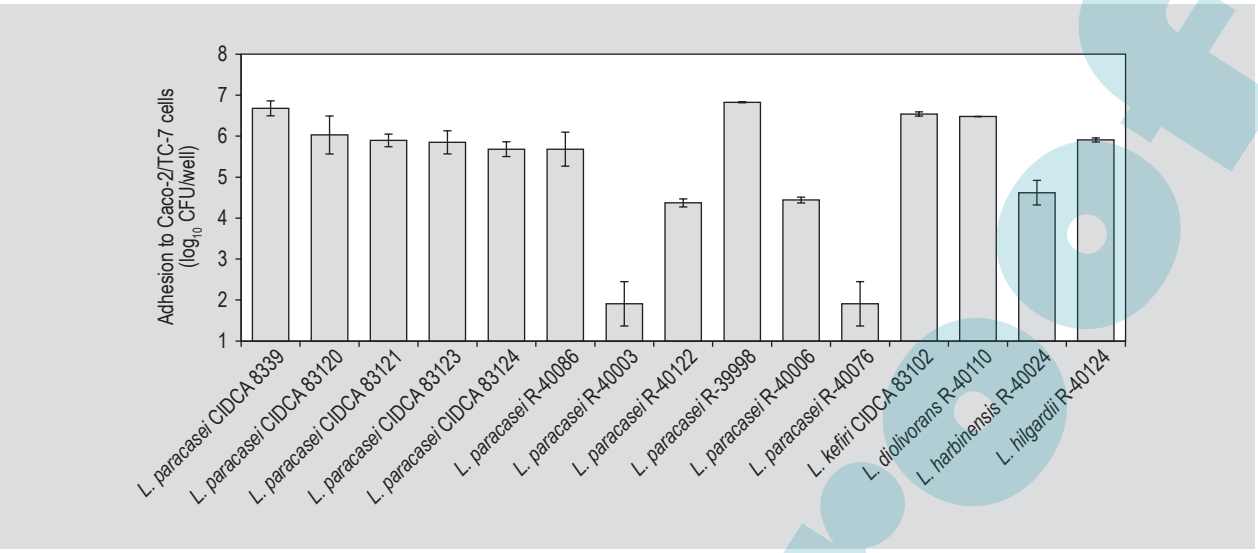

Figure 1. Lactobacilli adhesion to Caco-2/TC7 cells. Adhesion of lactobacilli isolated from sugary and milk kefir to Caco-2/TC-7 cells. Values were obtained by calculating the ratio of adhering lactobacilli (log_{10} cfu/well) ± standard deviation of three independent assays.

Table 2. Coaggregation of *Lactobacillus* strains with *Salmonella enterica* serovar Enteritidis (CC) and *Lactobacillus* auto-aggregation coefficient (AC).

Strain	% CC with *Salmonella*[1]		% AC[2] (1 h)
	1 h	20 h	
R-40086	0.2±0.1	2.2±0.1	6.0±0.4
R-40003	4.3±0.1	34.0±0.1	10.0±0.6
R-40122	5.5±0.1	32.2±0.1	0.2±0.1
R-39998	0.3±0.1	1.3±0.5	4.2±0.3
R-40006	5.2±0.1	31.1±0.1	1.3±0.4
R-40076	11.2±0.1	43.5±0.1	13.0±0.3
R-40110	1.0±0.1	1.0±0.3	14.6±0.3
R-40024	3.4±0.1	46.5±0.1	0.6±0.5
R-40124	0.4±0.1	1.4±0.1	8.6±0.5
CIDCA 83120	29.2±0.8	39.7±0.5	4.0±0.2
CIDCA 83121	33.0±0.3	52.3±2.3	3.0±0.3
CIDCA 83123	31.4±0.5	41.7±0.7	3.0±0.7
CIDCA 83124	39.2±1.6	47.7±1.8	4.0±0.8
CIDCA 8339	32.0±2.3	62.6±2.8	4.3±0.1
CIDCA 83102	27.6±5.0	72.5±7.1	5.2±0.1

[1] Values are the mean of CC ± standard deviation obtained from three independent assays. CC was calculated at 1 and 20 h at 37 °C as indicated in materials and methods.

[2] Values are the mean of AC ± standard deviation obtained from three independent assays. AC was calculated at 1 h as indicated in materials and methods.

determined. While strains *L. paracasei* R-40086, R-39998, *L. hilgardii* R-40124 and *L. diolivorans* R-40110 did not protect the monolayers from *Salmonella* invasion, *L. paracasei* strains CIDCA 8339, CIDCA 83120, CIDCA 83121, CIDCA 83123, CIDCA 83124 and *L. kefiri* CIDCA 83102 significantly diminished the pathogen invasion ($P \leq 0.05$) (Figure 2). Strains that showed a protective effect against *Salmonella* invasion were selected to conduct subsequent assays to evaluate barrier effect against *Salmonella* association/invasion to enterocytes.

Barrier effect of *Lactobacillus* against *Salmonella* association/invasion to Caco-2/TC-7 cells

As shown above, *L. paracasei* strains CIDCA 8339, CIDCA 83120, CIDCA 83121, CIDCA 83123, CIDCA 83124 and *L. kefiri* CIDCA 83102 protected Caco-2/TC-7 cells from *Salmonella* invasion when these strains were allowed to coaggregate with the pathogen. Therefore, these strains were incubated with the intestinal cells previous to *Salmonella* infection to evaluate if the pathogen could adhere and/or invade the enterocytes.

L. paracasei strains CIDCA 8339, CIDCA 83123 and CIDCA 83124 and *L. kefiri* CIDCA 83102 diminished *Salmonella* association (Figure 3A) while only the strains *L. paracasei* CIDCA 8339 and *L. kefiri* CIDCA 83102 were able to diminish the invasion to the enterocytes compared to *Salmonella* control (Figure 3B).

As the F-actin cytoskeletal rearrangement is one of the steps accompanying *Salmonella* entry into epithelial cells, and taking into account that *L. paracasei* CIDCA 8339 and

Galley Proof

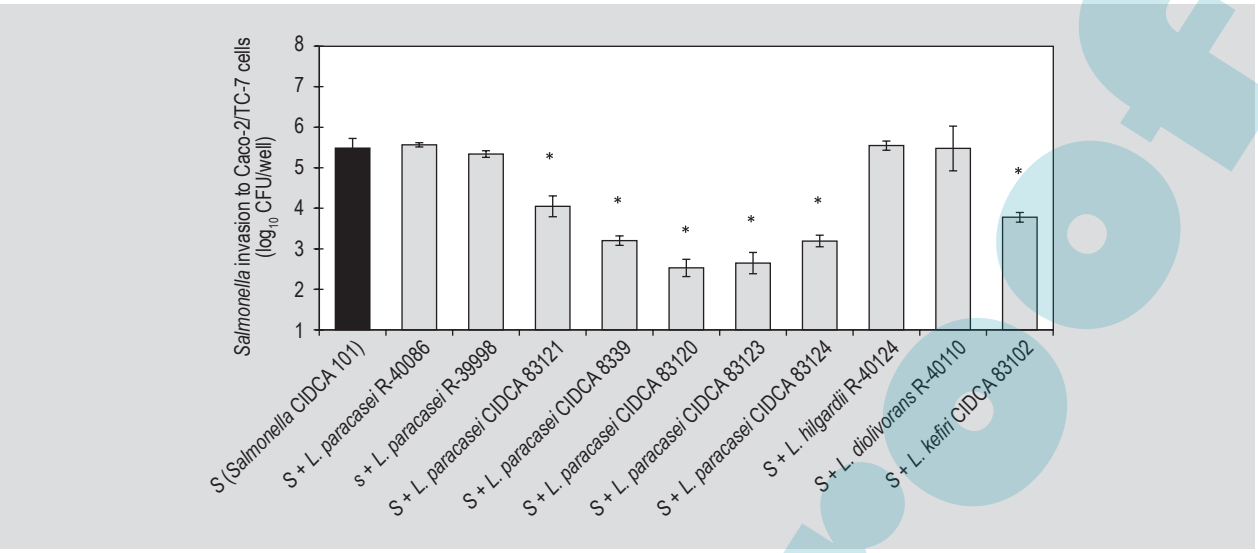

Figure 2. *Salmonella* invasion to Caco-2/TC7 cells after coincubation with *Lactobacillus* strains. *Salmonella* invasion (log_{10} cfu/well) to Caco-2/TC7 cells after the coincubation (1 h at 37 °C) of the pathogen with the selected strains *L. paracasei* (R-40086, R-39998, CIDCA 8339, 83120, 83121, 83123, 83124), *L. diolivorans* (R-40110), *L. hilgardii* (R-40124) and *L. kefiri* (CIDCA 83102). Results are the mean from 3 independent determinations ± standard deviation. * represent statistically significant differences (*P*≤0.05) compared to infection control (*Salmonella*).

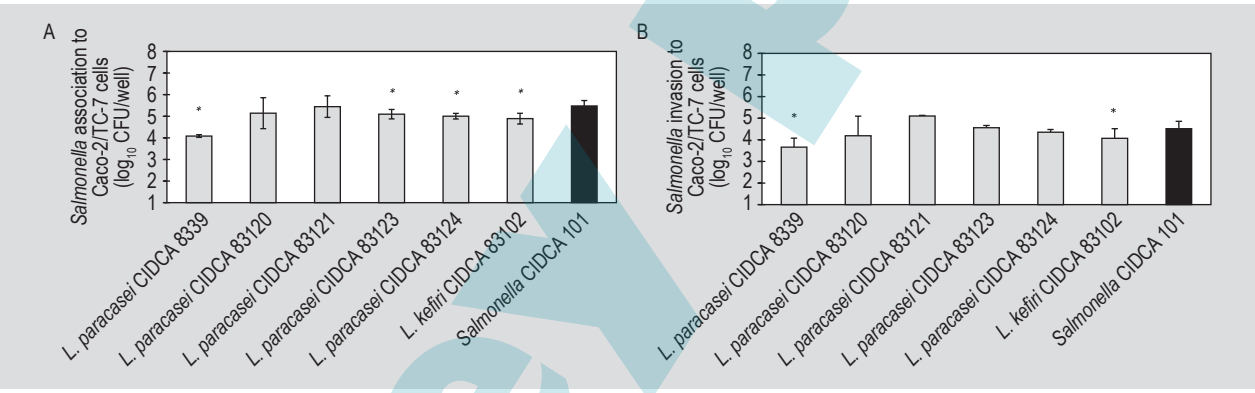

Figure 3. (A) *Salmonella* association. Barrier effect of *Lactobacillus* against *Salmonella* association. *Salmonella* association (log_{10} cfu/well) to Caco-2/TC7 cells after the coincubation of the eukaryotic cells with the selected strains *L. paracasei* (CIDCA 8339, 83120, 83121, 83123, 83124) and *L. kefiri* (CIDCA 83102). Results are the mean from 3 independent determinations ± standard deviation. * represent statistically significant differences (*P*≤0.05) compared to association control (*Salmonella*). (B) *Salmonella* invasion. Barrier effect of *Lactobacillus* against *Salmonella* invasion. *Salmonella* invasion (log_{10} cfu/well) to Caco-2/TC7 cells after the coincubation of the eukaryotic cells with the selected strains *L. paracasei* (CIDCA 8339, 83120, 83121, 83123, 83124) and *L. kefiri* (CIDCA 83102). Results are the mean from 3 independent determinations ± standard deviation. * represent statistically significant differences (*P*≤0.05) compared to invasion control (*Salmonella*).

L. kefiri CIDCA 83102 strains demonstrated to be able to diminish *Salmonella* invasion to enterocytes, cytoskeleton integrity was investigated.

Salmonella infection of Caco-2/TC-7 cells resulted in cytoskeleton disassembly (Figure 4B), showing characteristic dense spots of intense localised accumulations of F-actin, without detaching the cells to the surface of the well. Interestingly, the preincubation of the enterocytes with *L. kefiri* CIDCA 83102 and *L. paracasei* CIDCA 8339 strains (Figure 4C and 4D), resulted into an antagonist effect of cytoskeleton disorganisation elicited by the pathogen, preserving the integrity of the monolayers and showing a pattern similar to the control without treatment (Figure 4A).

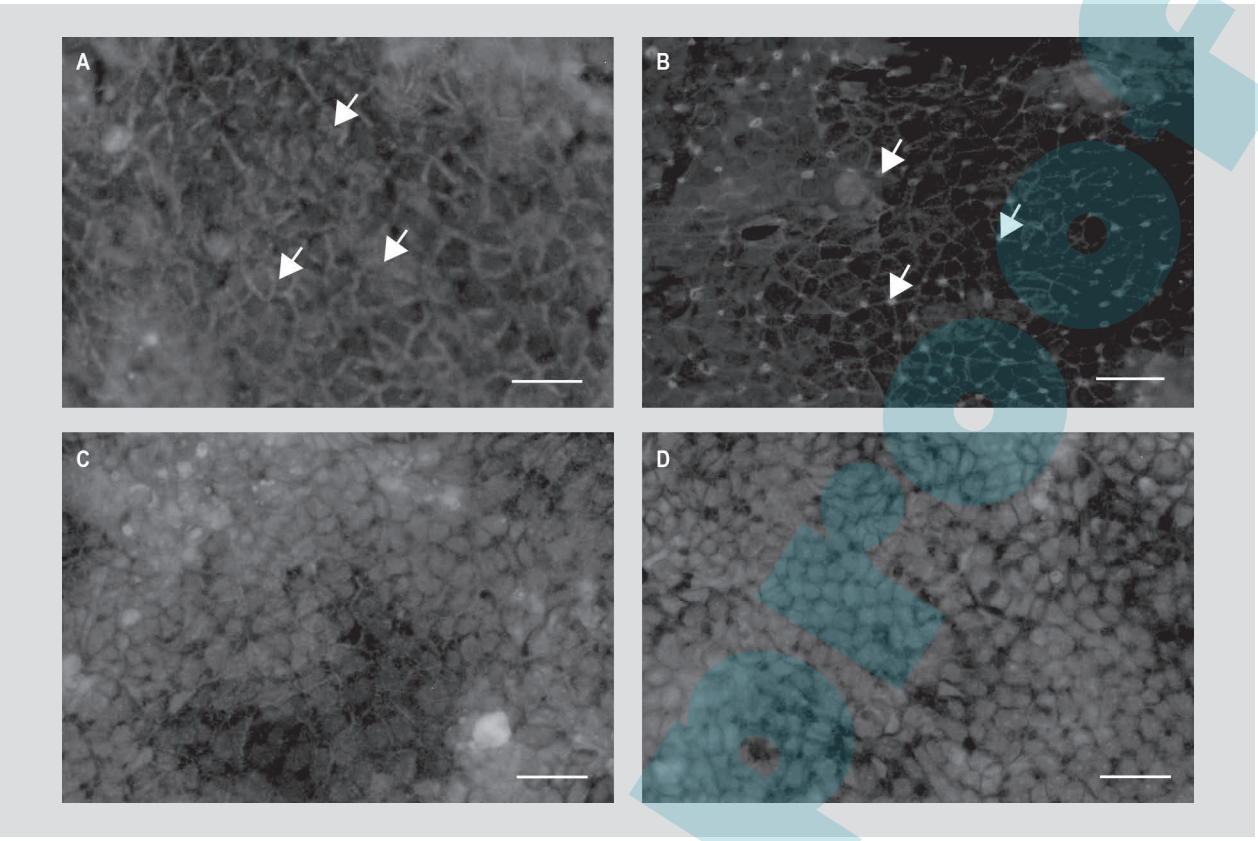

Figure 4. Cytoskeleton integrity. Micrographs showing localisation of F-actin in Caco-2/TC-7 cells. (A) Control uninfected cells treated with DMEM. The fine flocculated centrally located in the cells represents microvilli-associated F-actin (arrows). (B) Cells infected for 2 h with *Salmonella*. The localised dense spots of fluorescence (arrowheads) represent F-actin accumulation characteristically triggered by the pathogen. (C and D) Cells preincubated with *L. paracasei* strains CIDCA 8339 and CIDCA 83102, respectively, before *Salmonella* infection. Cells did not show the F-actin disassembly triggered by *Salmonella,* as no dense spots were observed. *Lactobacillus* incubation with Caco-2/TC7 cells showed an aspect similar to control cells without treatment (data not shown). Bar: 10 µm.

4. Discussion

For the development of novel probiotic foods the selection and evaluation of new and more specific probiotic microorganisms, that provides health benefits in the human population and improve certain functional disorder, is a continuous challenge. Although commonly the origin of these strains is the human gut, traditional fermented foods are an alternative source. In particular, *Lactobacillus* seems to possess the functional criteria required for probiotic candidates, i.e. cell adherence, coaggregation and antagonistic activity against *Salmonella* and technological features that allow applications in food industry (Foligné *et al.,* 2013). In this paper, we evaluated the probiotic properties of 15 *Lactobacillus* strains isolated from sugary and milk kefir.

Probiotic action could be associated with several mechanisms such as: production of antimicrobial substances, competitive exclusion, modulation of the immune response, alteration of intestinal bacterial metabolic activity, beneficial alteration of microecology in the human intestine, and inhibition of bacterial translocation (Servin, 2004). Many of the cited mechanisms could be related to bacterial surface properties (Cesena *et al.,* 2001; Pérez *et al.,* 1998; Polak-Berecka *et al.,* 2014). Auto and coaggregation as well as adhesion to intestinal epithelial cells are among the most important surface-related characteristics of probiotics. They are conditioned by a plethora of factors such as surface teichoic and lipoteichoic acids or S-layer proteins (Schär-Zammaretti and Ubbink, 2003), and exopolysaccharide synthesis (Lebeer *et al.,* 2010; Walter *et al.,* 2008) that may positively or negatively affect adherence to other cells (Goh and Klaenhammer, 2010; Lahtinen and Ouwehand, 2009; Polak-Berecka *et al.,* 2014).

Coaggregation properties of probiotic strains with pathogens may also be considered as desirable because bacterial clusters could hinder the movement of the pathogens through the mucus, preventing them to reach and colonise the epithelial cells (Collado *et al.,* 2008; Gueimonde *et al.,* 2006). In addition, probiotic microorganisms with the

ability to coaggregate with pathogenic bacteria may have an advantage over non-coaggregating because pathogens can be trapped by the probiotic bacteria and could be more easily removed from the intestinal environment preventing a possible infection by pathogenic bacteria (Boris *et al.,* 1997; Del Re *et al.,* 1998). Different surface molecules are involved in bacteria interactions (Tareb *et al.,* 2013), in consequence auto and coaggregation are strain-specific properties (Collado *et al.*, 2008). Results were in concordance with previous studies which demonstrated that autoaggregation values could be higher, similar (Tareb *et al.*, 2013; Xu *et al.*, 2009), or lower (Ferreira *et al.*, 2011) than coaggregation.

In the present study, when *Lactobacillus* strains were co-incubated for 1 h with *Salmonella* prior to Caco-2/TC7 cells infection, it was observed that the strains with coaggregation coefficients higher than 27% significantly reduced the invasion of this pathogen. In contrast, non-coaggregating strains had no effect on *Salmonella* invasion. These results suggest that the *Lactobacillus*-pathogen interaction begins before both microorganisms interact with the intestinal epithelium. As a result of this interaction, lactobacilli may create a microenvironment that could affect *Salmonella* virulence factors expression. Likewise, Golowczyc *et al.* (2007) previously observed that co-incubation of *Salmonella* with coaggregating *L. kefiri* strains significantly decreased the adhesion and invasion ability of this pathogen to Caco-2/TC-7 cells, and this was not observed with non-coaggregating *L. kefiri* strains. Valeriano *et al.* (2014) reported a positive correlation between coaggregation and displacement of *Escherichia coli*. However, the same authors concomitantly found a correlation between adhesion, aggregation and hydrophobicity, whereas no such correlation was observed between these parameters in the present study. Cell surface hydrophobicity is one of the most important factors controlling adhesion of microorganisms to surfaces (Schillinger *et al.,* 2005). According to Otero *et al.* (2004) bacterium classified as hydrophobic can be considered as able to mediate adhesion. In our work no correlation was observed between adhesion and hydrophobicity. Only one strain showed a high percentage of hydrophobicity (*L. diolivorans* R-40110) and proved to have a high adhesion compared with the other strains. However, many other strains with high adhesion were hydrophilic.

Thus, different mechanisms can be utilised by probiotic strains to exert an inhibitory effect against *Salmonella*: adhesion to the intestinal epithelium (and thus create a barrier effect and prevent pathogen adhesion or invasion) or through a coaggregation effect with the pathogen. Our results showed that some strains with high adhesion capacity were non-coaggregating (i.e. R-39998), while other strains with high percentages of coaggregation showed variable adhesion capacity (CIDCA 83120, 83121, 83123, 83124, 8339, 83102). These are independent mechanisms that could contribute to inhibit the adhesion and invasion of *Salmonella*.

In addition to nutrient absorption, the gut barrier is also responsible for the organisation of host defence mechanisms against harmful macromolecules and pathogen invasion. In this study, strains with the highest coaggregation coefficients with *Salmonella* and with a high or medium ability to adhere to epithelial cells were selected to assess their potential contribution to the gut barrier. Four out of the six coaggregating strains were able to reduce association with *Salmonella*, and only 2 strains (i.e. *L. paracasei* CIDCA 8339 and *L. kefiri* CIDCA 83102) were able to reduce *Salmonella* invasion to intestinal cells being this two strains the ones that had the highest adhesion ability. These results are in concordance to previous reports which comments that the inhibition of *Salmonella* sp. adhesion to Caco-2 cells *in vitro* was directly related with the adhesion ability of the *Lactobacillus* strains (Fernandez *et al.,* 2003; Lee *et al.,* 2003; Santos *et al.,* 2003).

F-actin network disassembly is considered as one of the main mechanisms by which pathogenic microorganisms structurally and functionally alter the host cells, and this has also particular relevance for intestinal epithelial barrier maintenance (Bernet-Camard *et al.,* 1996; Medrano *et al.,* 2009). By this strategy, pathogens could associate to cell surface or even invade them. In the present study, we demonstrated that strains *L. paracasei* CIDCA 8339 and *L. kefiri* CIDCA 83102 were able to diminish *Salmonella* invasion and we also demonstrated an antagonistic effect on cytoskeleton disruption elicited by the pathogen. This may result from the blocking of the initial crosstalk between *Salmonella* and Caco-2/TC-7 cell effectors. By coaggregating with the pathogen, or by direct association to the intestinal cells, these lactobacilli can protect eukaryotic cells from *Salmonella* invasion and thus diminish cytoskeleton disassembly. *Lactobacillus* protection against cytoskeleton disorganisation elicited by *Salmonella* in similar *in vitro* models was previously reported (Coconnier *et al.,* 2000; Li *et al.,* 2011). Recently, Yu *et al.* (2015) found that *Lactobacillus fructosus* C2 was able to antagonise *Salmonella* Typhimurium alterations on Caco-2 monolayers tight junctions, while Koninkx *et al.* (2010) found that probiotic strains of *Lactobacillus* sp. stabilised the transepithelial barrier integrity of Caco-2 cells when they were preincubated before *Salmonella enterica* infection. These mechanisms should not be ruled out as an alternative pathway for the observed antagonism on cytoskeleton disruption and/or pathogen invasion.

In our research group, it was demonstrated that *L. paracasei* CIDCA 8339 produces high molecular weight exopolysaccharides affecting the viscosity of the fermented milk (Hamet *et al.,* 2015). Exopolysaccharide production

could also be related to the surface properties of probiotic strains (Lebeer *et al.,* 2010; Walter *et al.,* 2008), and this could explain the results obtained with this particular strain. This strain could be valuable candidate for texture improvement of fermented milk products. The ability to produce exopolysaccharides together with the results demonstrated in this work suggest that *L. paracasei* CIDCA 8339 is a good candidate as a probiotic microorganism and would be feasible to incorporate into a food matrix.

5. Conclusions

Our results suggest that *L. kefiri* strain CIDCA 83102 and *L. paracasei* strain CIDCA 8339 isolated from milk kefir could be considered as appropriate probiotic candidates. Both strains showed adhesion ability to enterocytes, inhibited *Salmonella* association/invasion and prevented cytoskeleton disorganisation on *in vitro* assays. Both strains, alone or in combination with other probiotics, may prove useful in future applications. Furthermore, being food isolated strains they are pre-adapted to the food environment, which might be more feasible for their incorporation into healthy functional foods. Future studies will be addressed on the ability of these lactobacilli strains to inhibit infection by other enteropathogens, as well as their role of evoking beneficial effects in animal models. More studies should be conducted for the inclusion of these selected strains into functional foods.

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