International Dairy Journal 33 (2013) 22–26

Contents lists available at SciVerse ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

Lactobacillus plantarum isolated from kefir: Protection of cultured Hep-2 cells against *Shigella* invasion

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ARTICLE INFO

Article history:
Received 28 February 2013
Received in revised form
30 May 2013
Accepted 3 June 2013

ABSTRACT

Two types of microbial mixtures containing selected lactic acid bacteria and yeasts isolated from kefir grains were tested against the *Shigella* invasion of Hep-2 cells in culture. A five-strain mixture demonstrated a significant inhibition of the cell internalisation of *Shigella flexneri* and *Shigella sonnei*. The addition of single kefir strains or their mixtures to the cells, before the addition of *Shigella*, protected more efficiently than the simultaneous addition of the lactic-acid bacteria and *Shigella* on the cells. Among the kefir strains assayed, *Lactobacillus plantarum* strain CIDCA 83114 showed the most significant inhibition of the invasion. Both the cell walls of *L. plantarum* and the intact bacterial cells demonstrated an equivalent protection of cell monolayers; with the possibility of a protein or peptide mediating this effect. These results provide evidence for the potential inhibitory properties of certain kefir strains, such as *L. plantarum* CIDCA 83114, against disease-producing species of *Shigella*.

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1. Introduction

Probiotic species of the lactobacilli, bifidobacteria and yeasts have demonstrated significant health benefits, including the protection of a potential host against infectious diseases caused by enteric pathogens and the prevention of intestinal disorders (Gupta & Garg, 2009). The ability to adhere to the mucosal surfaces along with antagonism towards gastrointestinal pathogens has become desirable criteria for selection of probiotic microorganisms (Vasiljevic & Shah, 2008).

Shigella is an enteric Gram-negative bacillus causing a dysenteric syndrome in humans and is one of the most frequent causes of acute diarrhoea in developing countries. *Shigella flexneri* and *Shigella sonnei*, accordingly, are often the species identified in children with bacillary dysentery (Merino, Hreñuk, Ronconi, & Alonso, 2004; Xia et al., 2011). The pathogenesis of this infection begins with an invasion of the colon epithelium followed by intracellular bacterial replication and spread into adjacent cells (Watarai, Tobe, Yoshikawa, & Sasakawa, 1995). One of the crucial processes in cellular uptake of *Shigella* is an actin polymerisation leading to membrane ruffling, a cytoskeletal rearrangement that occurs during cellular internalisation of *Shigella* (Adam, Arpin, Prévost, Gounon, & Sansonetti, 1995). Hep-2 cells in culture have been used to study *Shigella* invasion along with the subsequent events that lead to an efficient bacterial colonisation of the epithelial monolayer (Bukholm, Modalsli, & Degré, 1986; Day, Scotland, & Rowe, 1981).

Kefir grains are a source of both bacteria and yeasts having potential probiotic properties (Chifiriuc, Cioaca, & Lazar, 2011; Farnworth, 2005; Garrote, Abraham, & De Antoni, 2001; Lopitz-Otsoa, Rementeria, Elguezabal, & Garaizar, 2006), while certain of the kefir bacterial isolates have been reported capable of inhibiting foodborne pathogens, such as *Salmonella*, *Staphylococcus aureus* and *Escherichia coli* (Raja, Gajalakshmi, Raja, & Imran, 2009; Santos, San Mauro, Sanchez, Torres, & Marquina, 2003).

The antimicrobial and immunomodulatory properties of strains isolated from CIDCA kefir grains have been reported. For example, in culture *Lactobacillus kefiri* CIDCA 8348 protected epithelial cells against *Salmonella* invasion (Golowczyc, Mobili, Garrote, Abraham, & De Antoni 2007), while *Lactococcus lactis* subsp. *lactis* CIDCA 8221 were observed to secrete heat-sensitive metabolites that protected

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0958-6946/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.idairyj.2013.06.004

eukaryotic cells from the cytopathic effects caused by *Clostridium difficile* toxins (Bolla, Carasi, Serradel, & De Antoni 2013). Furthermore, *Lactobacillus plantarum* CIDCA 83114 exhibited an antimicrobial activity in spot assays against *Salmonella enterica* serovar Typhimurium and *S. sonnei* (Golowczyc et al., 2008) and decreased the adhesion of enterohaemorraghic *E. coli* to Hep-2 cells, thus protecting them from injury (Hugo, Kakisu, De Antoni, & Perez, 2008). Among the kefir yeasts, *Kluyveromyces marxianus* CIDCA 8154 and *Saccharomyces cerevisiae* CIDCA 8112 inhibited the innate response of the intestinal epithelium triggered by different proinflammatory pathways through a mechanism dependent on a modulation of the necrosis factor-κB (Romanin et al., 2010). Finally, we have recently reported that a freeze-dried five-strain microbial mixture, containing the five above-mentioned kefir strains, inhibited the growth of *S. sonnei* cultures in vitro (Bolla, Serradell, de Urraza, & De Antoni, 2011), while a microbial mixture containing *Lb. plantarum* CIDCA 83114 together with *Streptococcus thermophilus* antagonised cytopathogenesis by enterohaemorragic *E. coli* Shiga toxin in cultures of Vero cells (Kakisu, Abraham, Tironi Farinati, Ibarra, & De Antoni, 2013).

Very few studies have been carried out on the effects of probiotic lactobacilli against *Shigella* or on the impact of kefir microorganisms in particular on the protection of epithelial cells against this pathogen. Moorthy, Murali, and Niranjali Devaraj (2010) reported that a pretreatment of Caco-2 cells with a combination of *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* before the addition of *Shigella dysenteriae* was a better method against the invasion of the bacteria than the approach of competitive exclusion through the simultaneous addition of the probiotics. These lactobacilli, furthermore, when incubated in combination, were found to act synergistically in their antagonism to *Shigella* internalisation.

In view of this background information, the aim of this study was to evaluate the ability of two kefir multistrain mixtures, along with the effectiveness of individually selected bacterial isolates from those mixtures, to inhibit the invasion of Hep-2 cells in culture by *S. flexneri* and *S. sonnei*.

2. Materials and methods

2.1. Microorganisms and culture conditions

The pure strains isolated from kefir grains comprised: *Lc. lactis* CIDCA 8221, *Lb. plantarum* CIDCA 83114, *Lb. kefiri* CIDCA 8348, *Kluyveromyces marxianus* CIDCA 8154 and *Sac. cerevisiae* CIDCA 8112. These strains were previously identified and characterised by Delfederico et al. (2006) and Garrote et al. (2001). Both the lactobacilli and the yeasts were grown in MRS-broth (Difco, Sparks, MD, USA) for 24 or 48 h at 30 °C. *Lc. lactis* was grown in 1.1.1 growth media containing 1% (w/v) of tryptone (Difco) plus 1% (w/v) of yeast extract (Biokard Diagnostic, Beauvais, France) plus 1% (w/v) lactose (Mann Research Laboratories, New York, NY, USA) (Abraham, De Antoni, & Añon, 1990) for 24 h at 30 °C. *Str. thermophilus* CIDCA 321, isolated from yoghurt (Perez, De Antoni, & Añon 1991), was grown in MRS for 24 h at 37 °C. The kefir and *Shigella* strains were stored frozen at −80 °C in 50% (w/v) milk and 0.6 M sucrose solution, respectively, and used for experiments in the second passage in the corresponding media. *S. sonnei* strain 45 and *S. flexneri* strain 72, obtained from the Sor María Ludovica Interzonal Hospital (La Plata, Argentina), were cultured in tryptic-soy broth (Biokard Diagnostic, Beauvais, France) at 37 °C with shaking for 18 h.

2.2. Preparation of strain mixtures

Two mixed cultures were prepared: one containing 10^8 cfu mL^{-1} of *Lb. plantarum* CIDCA 83114 and 10^7 cfu mL^{-1} of *Str. thermophilus* CIDCA 321, to be referred to as the two-strain mixture, and the other consisting of *Lb. plantarum* CIDCA 83114, *Lb. kefiri* CIDCA 8348, *Lc. lactis* CIDCA 8221, *K. marxianus* CIDCA 8154 and *Sac. cerevisiae* CIDCA 8112, to be designated as the five-strain mixture. The final concentration of bacteria and yeasts in the five-strain mixture was 10^9 cfu mL^{-1} and 10^6 cfu mL^{-1}, respectively. Each microorganism was also grown individually under the conditions described above, and stationary-phase cultures of each one were harvested by centrifugation at 10,000 × *g* for 10 min and resuspended in the same volume of fresh medium.

2.3. Cell culture

Hep-2 cells were cultured in Dulbecco's Modified Eagle's Minimum Essential Medium (DMEM; GIBCO BRL Life Technologies, Rockville, MD, USA) supplemented with 10% (v/v) of foetal bovine serum (PAA Laboratories, GmbH, Pasching, Austria), 1% (v/v) nonessential amino acids (GIBCO BRL Life Technologies), and antibiotics (12 IU mL^{-1} penicillin and 12 μg mL^{-1} streptomycin, GIBCO BRL Life Technologies). Cells were inoculated (2.5 × 10^5 cells per well) into 24-well tissue-culture plates (Greiner Bio One, Frickenhausen, Germany) and incubated at 37 °C for 48 h in an atmosphere of 5% CO_2–95% (v/v) air.

2.4. Determination of bacterial invasion and the action of kefir microorganisms

The ability of *Shigella* to invade cell monolayers and the potential protection of multistrain mixtures or their individual microorganisms were evaluated in Hep-2 cells as a model of microorganism invasion.

The *Shigella*-invasion assay was evaluated through the following two experimental designs for invasiveness before determining the number of internalised bacteria in Hep-2 cells by agar-plate counting.

Coincubation: The multistrain mixture or their single microorganisms and 10^8 cfu mL^{-1} of *Shigella* were suspended in serum-free DMEM medium and subsequently incubated together with shaking for 1 h at 37 °C. The bacteria were then inoculated onto a cell monolayer and incubated again for 2.5 h at 37 °C in a controlled atmosphere of 5% (v/v) CO_2.

Preincubation: The multistrain mixture or their single microorganisms were incubated on cell monolayers for 1 h at 37 °C, in a cell-culture incubator followed by a 1 h incubation at 37 °C, after the addition of 10^8 cfu mL^{-1} of *Shigella*.

In all experiments the *Shigella* cells internalised were determined after the incubation in the following manner. The monolayer was washed with phosphate buffered saline (PBS). To each well 1 mL of DMEM with gentamicin was added (100 μg mL^{-1}) and the monolayer incubated for 1.5 h at 37 °C to remove bacteria adhering to the surface. The cell monolayer was washed again and the cells lysed by incubation in 1 mL of sterile distilled water for 1 h at 37 °C. Finally, after homogenisation of the cells by pipetting, the contents of each well were removed for performing counts of the viable internalised microorganisms by colony formation in nutrient agar during subsequent 24-h incubation at 37 °C.

2.5. Scanning electron microscopy

The microscopical analysis of the cell cultures was performed on glass coverslips for cell cultures (Assistent, Glaswarenfabrik Karl Hetch KG, Sondheim, Germany). The infected monolayers were fixed for 1 h at 37 °C with 1% (v/v) glutaraldehyde (Riedel de Haen, Seelze, Germany) at 4 °C for 3 h. Smears were dehydrated in a graded series of ethanol solutions. Finally, the samples were critical-point dried by a stream of CO_2 (Model CP30, Baltec), gold

coated (Jeol FineCoat Sputter JFC-1100, Jeol Ltd., Akishima Tokyo, Japan) and then examined with a Jeol model JSM-T100 scanning electron microscope (Jeol Ltd.).

2.6. Proteolytic enzyme treatment of lactobacilli

Pepsin (Sigma, St. Louis, MO, USA) was prepared in glycine-HCl 50 mM, NaCl 100 mM, pH 2.2 at a concentration of 2.5 mg mL^{-1}. For pepsin treatment using this enzyme preparation, 1 mL of bacterial suspension (10^9 cfu mL^{-1}) was centrifuged at $10,000 \times g$ for 10 min and the pellets were resuspended in the same volume of the enzyme solution, and finally incubated for 1 h at 37 °C. To avoid cell detachment of the monolayer, the enzyme solution was removed and inactivated at pH 7 with 1 mL of PBS after fulfilling its proteolytic function on lactobacilli.

2.7. Preparation of cell wall extracts

The *Lactobacillus* culture obtained from 1 L of MRS culture was centrifuged at $10,000 \times g$ for 10 min and washed with PBS. The pellet was lysed mechanically at −20 °C with a French Press XS-17523 (AB Biox, Järfälla, Sweden) by three consecutive disruptions at 100 kN. The disrupted suspension was centrifuged for 10 min at $10,000 \times g$ and 4 °C and the resulting supernatant was then ultracentrifuged at $35,000 \times g$ and 4 °C (TL Optima, Beckmann Instruments Inc., Palo Alto, CA, USA). The final pellet was washed with PBS to a constant OD at 280 nm and then suspended in 1 mL of PBS for storage at −20 °C.

2.8. Statistical analysis

All experiments were performed at least three times. The data shown are the means ± the standard errors. Analysis of variance (ANOVA) at 95% confidence intervals was run in order to reveal possible differences between the samples. The ANOVA was performed with the SPSS statistical package version 15.0 (SPSS Inc., Chicago, IL, USA). The statistical significance of the differences was determined by Dunnett's post hoc test, with $P \leq 0.05$ as the threshold of confidence.

3. Results and discussion

The addition of *S. flexneri*, after 1 h of preincubation with kefir strains on cell monolayers (preincubation protocol), led to a lower concentration of internalised pathogens compared with the cell assay with the strains and *Shigella* being added at the same time (coincubation protocol; Table 1). A drastic diminution in the invasiveness was obtained with *Lb. plantarum* CIDCA 83114 since an extremely low percentage of *Shigella* became internalised (only ≤5 percent); whereas in contrast, *Lb. kefiri* CIDCA 8348 did not exert any significant protection. Under the condition of coincubation, the lactococci and yeasts caused less protection, if any, than *Lb. plantarum* although with preincubation appreciable inhibition by these strains did occur. In accordance with our results, Moorthy et al. (2010) also demonstrated that the incubation of the cells with lactobacilli prior to the addition of *Shigella* was the best treatment against the invasion.

The two-strain- and five-strain-mixtures of bacteria and yeast cited above were effective in reducing the invasion of *S. flexneri* and *S. sonnei* under the preincubation protocol (Table 2). The five-strain mixture provided a better protection than did the two-strain mixture, while the pronounced effect of preincubation with *Lb. plantarum* alone indicated that strain CIDCA 83114 in particular was strongly associated with the protection against *Shigella* internalisation, either alone or in combination with the other probiotic strains. Moreover, this inhibition of *Shigella* invasiveness was greater at the higher concentration of *Lb. plantarum*. That the greatest antagonism to *Shigella* internalisation occurred with the five-strain mixture furthermore suggests a combined action on the part of the kefir microorganisms.

Scanning electron microscopy gave an overall view of the morphologic changes occurring on the surface of Hep-2 cells after infection with *S. flexneri* 72 (Fig. 1). *Shigella* invasion resulted in a removal of the microvilli, cellular retraction and the formation of protrusions extending along the surface of the cells (Fig. 1B). *Shigella* internalisation into epithelial cells has been found to trigger a cascade of transmembrane and intracellular signals producing a disruption of the actin network and leading to major cytoskeletal rearrangements (Adam et al., 1995; Clerc & Sansonetti, 1987). Pretreatment of cells with the five-strain mixture, however, protected the monolayer against deformation of the cells by *Shigella* infection and preserved the normal morphology of Hep-2 cells, and by implication, the structure of the cytoskeletal elements on the cell surface (Fig. 1C). Fig. 1A furthermore demonstrates that the incubation of Hep-2 cells with the five-strain mixture per se produced no alteration in the cell monolayer.

To understand the mechanism underlying the reduction of *Shigella* internalisation upon lactobacilli pretreatment, the role of the cell walls and the surface proteins of *Lb. plantarum* CIDCA 83114 were tested. When Hep-2 cells were preincubated with *Lb. plantarum* CIDCA 83114 cell walls (Fig. 2, second column), in an amount

Table 1
Shigella flexneri invasion of Hep-2 cells either coincubated or preincubated with lactobacilli or yeasts isolated from kefir.[a]

Protocol	Percentage of *Shigella flexneri* 72 internalised in Hep-2 cells	
	Coincubation assay	Preincubation assay
Shigella control	100[a]	100[a]
Lb. plantarum CIDCA 83114	5.0 ± 1.3[c]	3.7 ± 3.3[c]
Lb. kefiri CIDCA 8348	99.0 ± 3.9[a]	68.4 ± 5.3[b]
Lc. lactis CIDCA 8221	97.0 ± 9.4[a]	42.6 ± 10.5[bc]
Sac. cerevisiae CIDCA 8112	89.3 ± 6.4[a]	45.3 ± 5.6[bc]
K. marxianus CIDCA 8154	88.6 ± 5.3[a]	32.2 ± 15.9[bc]

[a] The concentration of *S. flexneri* inoculated and internalised in the control cells was 1.23×10^8 cfu mL^{-1} and 1.9×10^5 cfu mL^{-1} respectively; with the latter value being considered as 100 percent. Different superscript letters in the same row and column indicate significant differences between the mean values according to analysis of variance (ANOVA) at a 95% confidence interval.

Table 2
Shigella invasion of Hep-2 cells after preincubation with either mixtures of strains or *Lactobacillus plantarum* CIDCA 83114.[a]

Protocol	Percentage of *Shigella* internalised in Hep-2 cells after preincubation with kefir microorganisms	
	Shigella flexneri 72	*Shigella sonnei* 45
Shigella control	100[a]	100[a]
Five-strain mixture (*Lb. plantarum*, *Lb. kefiri*, *Sac. cerevisiae*, *K. marxianus*, *Lc. lactis*)	$0.04 \pm 6.9 \times 10^{-3}$[c]	$0.06 \pm 9.2 \times 10^{-3}$[c]
Two-strain mixture (*Lb. plantarum* and *Str. thermophilus*)	8.3 ± 0.8[b]	7.1 ± 0.3[b]
Lb. plantarum CIDCA 83114 (10^9 cfu mL^{-1})	3.7 ± 3.3[b]	8.4 ± 1.6[b]
Lb. plantarum CIDCA 83114 (10^8 cfu mL^{-1})	12.7 ± 2.6[b]	8.7 ± 0.7[b]

[a] The concentration of *S. flexneri* 72 and *S. sonnei* 45 internalised in the control was 1.9×10^5 cfu mL^{-1} and 4.3×10^3 cfu mL^{-1}, respectively; these values have been considered as 100 percent of internalisation. Different superscript letters in the same row and column indicate significant differences between the mean values according to analysis of variance (ANOVA) at a 95% confidence interval.

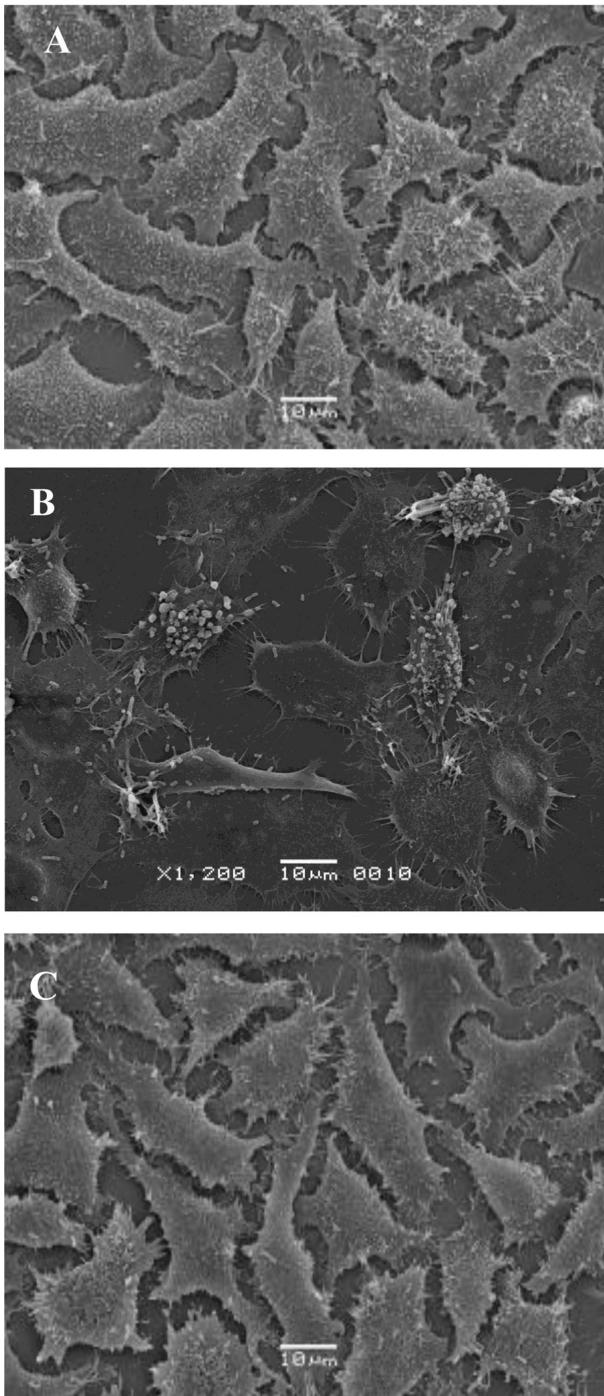

Fig. 1. Scanning electron microscopy (×1200) of Hep-2 cells: (A) incubated with 10^9 cfu mL^{-1} of the five-strain mixture, (B) incubated for 2.5 h with 10^8 cfu mL^{-1} of *Shigella flexneri* 72, (C) preincubated with 10^9 cfu mL^{-1} five-strain mixture and then incubated for 2.5 h with 10^8 cfu mL^{-1} of *S. flexneri* 72.

equivalent to the concentration of intact lactobacilli present in the five-strain mixture (Fig. 2, first column), the number of *Shigella* internalised decreased by 70% for both species of infective agent. This protection increased at a higher concentration of cell walls (Fig. 2, third column). These results evidenced the involvement of cell walls in the mechanism of protection against the invasion of both *Shigella* strains. Treatment of whole *Lb. plantarum* cells with the protease pepsin led to a significant loss of the protection against the internalisation of pathogen (Fig. 2, fourth column), thus indicating that key peptides and/or proteins present on the surface of

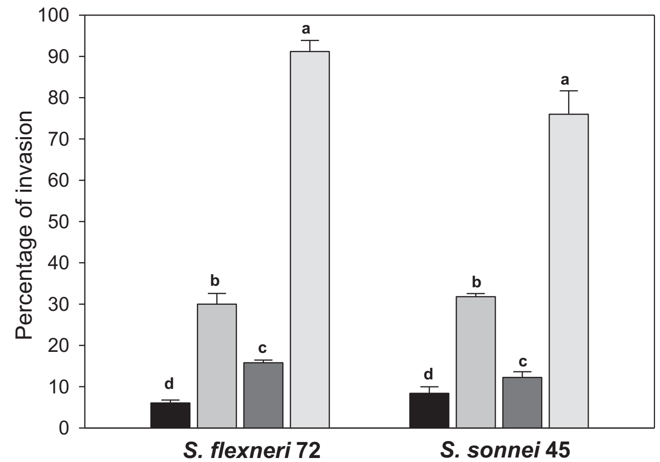

Fig. 2. Effects of *Lactobacillus plantarum* CIDCA 83114 or the bacterial cell walls on the invasion of Hep-2 cells by *Shigella flexneri* and *Shigella sonnei*. Percentage of *Shigella* internalised: after preincubation with 10^9 cfu mL^{-1} lactobacilli (■); after preincubation with lactobacilli cell walls equivalent to 10^9 cfu mL^{-1} (▨); after preincubation with lactobacilli cell walls equivalent to 10^{12} cfu mL^{-1} (▩); after preincubation with 10^9 cfu mL^{-1} lactobacilli treated with pepsin (□). Different superscripts indicate significant differences between the mean values according to analysis of variance (ANOVA) at 95% confidence intervals.

the strain CIDCA 83114 cells could be involved in the antagonism to *Shigella* invasion of Hep-2 cells.

Although not many studies have dealt with the involvement of the bacterial surface in providing protection against *Shigella* invasion, the ability of certain proteins to inhibit the bacterial internalisation has nevertheless been described in specific reports. Certain species of glycoproteins, e.g., the lectins, have been found to inhibit the invasion of Hep-2 cells by *S. dysenteriae* (Raja, Murali, Kumar, & Niranjali Devaraj, 2011). Willer, Lima, and Giugliano (2004) demonstrated that the anti-invasive effects are mediated by the binding of glycoproteins to *Shigella* surface proteins, thus implying that functional proteins expressed on the bacterium's surface may possibly constitute a specific target on *Shigella*. Certain lactobacilli have a proteinaceous layer on their surface referred to as the S layer; this structure has accordingly been associated with the protection against adhesion and invasion of *Shigella* and other pathogens (Golowczyc et al., 2007; Zhang et al. 2010). The results presented here, however, demonstrate that the inhibition of *Shigella* invasion by *Lb. plantarum* CIDCA cannot be related to S-layer proteins since this strain of *Lactobacillus* was found not to express these proteins (Garrote et al., 2004); while *Lb. kefiri* CIDCA 83113, whose external cell surface carries S-layer proteins (Garrote et al., 2004), conferred no protection at all against *Shigella* invasion (*cf.* Table 1). According to Boekhorst, Wels, Keerebezem, and Siezen (2006), the *Lb. plantarum* genome encodes anchored cell-surface proteins that are involved in adhesion, enzyme action, phage functions, and other still unknown properties. Since the expression of any one or more among a number of functional cell-wall proteins in the strain CIDCA 83114 may be involved in the antagonism to *Shigella* documented here, more advanced studies are necessary to explore the mechanism of this form of probiotic protection still further.

4. Conclusions

The results of this study demonstrate antagonism to the invasion of mammalian cells by *S. flexneri* and *S. sonnei* on the part of a five-strain mixture containing kefir bacteria and yeasts. These findings constitute the first evidence of *Shigella* anti-invasive protection of human Hep-2 cells by kefir strains. Since *Lb. plantarum* CIDCA 83114 – the most effective strain tested here – had

previously demonstrated an ability to protect mammalian cells against *E. coli*-supernatant cytotoxicity (Kakisu, Irigoyen, Torre, De Antoni, & Abraham, 2011) and now, has manifested a maximal inhibition of *Shigella* invasion, this strain has proven to be a promising candidate for inclusion in probiotic starter cultures. The relevance of the *Lb. plantarum* cell walls in blocking the host–pathogen interaction, likewise recorded here, provides the first evidence pointing to a possible explanation of the mechanism underlying *Lactobacillus* protection.

Acknowledgements

E. Kakisu and P. Bolla are fellows of the National Research Council of Argentina (CONICET, Argentina). G. L. De Antoni is a researcher of CIC-PBA; A. G. Abraham is researcher of CONICET. G. L. De Antoni, P. de Urraza and A. G. Abraham are Professors of the School of Sciences of the National University of La Plata (UNLP). This work was supported by the National Agency of Scientific and Technical Promotion (ANPCyT), CONICET, CIC-PBA, UNLP. Dr. Donald F. Haggerty, a native English speaker, edited the final version of the manuscript.

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