Available online at www.sciencedirect.com
ScienceDirect
International Journal of Food Microbiology 118 (2007) 264–273
INTERNATIONAL JOURNAL OF Food Microbiology
www.elsevier.com/locate/ijfoodmicro

Protective action of *Lactobacillus kefir* carrying S-layer protein against *Salmonella enterica* serovar Enteritidis

M.A. Golowczyc [a], P. Mobili [a], G.L. Garrote [a], A.G. Abraham [a], G.L. De Antoni [a,b,*]

[a] *Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), Argentina*
[b] *Cátedra de Microbiología, Facultad de Ciencias Exactas, UNLP Calle 47 y 116, 1900-La Plata, Argentina*

Received 16 July 2007; accepted 23 July 2007

Abstract

Eight *Lactobacillus kefir* strains isolated from different kefir grains were tested for their ability to antagonize *Salmonella enterica* serovar Enteritidis (*Salmonella enteritidis*) interaction with epithelial cells. *L. kefir* surface properties such as autoaggregation and coaggregation with *Salmonella* and adhesion to Caco-2/TC-7 cells were evaluated. *L. kefir* strains showed significantly different adhesion capacities, six strains were able to autoaggregate and four strains coaggregated with *Salmonella.*

Coincubation of *Salmonella* with coaggregating *L. kefir* strains significantly decreased its capacity to adhere to and to invade Caco-2/TC-7 cells. This was not observed with non coaggregating *L. kefir* strains.

Spent culture supernatants of *L. kefir* contain significant amounts of S-layer proteins. *Salmonella* pretreated with spent culture supernatants (pH 4.5–4.7) from all tested *L. kefir* strains showed a significant decrease in association and invasion to Caco-2/TC-7 cells. Artificially acidified MRS containing lactic acid to a final concentration and pH equivalent to lactobacilli spent culture supernatants did not show any protective action. Pretreatment of this pathogen with spent culture supernatants reduced microvilli disorganization produced by *Salmonella*. In addition, *Salmonella* pretreated with S-layer proteins extracted from coaggregating and non coaggregating *L. kefir* strains were unable to invade Caco-2/TC-7 cells. After treatment, *L. kefir* S-layer protein was detected associated with *Salmonella*, suggesting a protective role of this protein on association and invasion.

Keywords: *Lactobacillus kefir*; S-layer; Probiotic; *Salmonella* sp.

1. Introduction

Salmonella is one of the most extensively studied and characterised bacterial pathogens. This microorganism causes a variety of disease syndromes such as enteric fever, bacteriemia, focal infections and enterocolitis. *Salmonella enterica* serovar Enteritidis (*Salmonella enteritidis*), carried by chickens and poultry products, is one of the major sources of human intestinal infections (Patrick et al., 2004; Velge et al., 2005).

Salmonella species can adhere to and invade a wide variety of cell types, and during interaction with eukaryotic cells they use different types of fimbriae and numerous proteins (Darwin and Miller, 1999). The ability to invade mammalian cells is critical to initiate the infection (Carlson and Jones, 1998).

Probiotic bacteria, mainly lactic acid bacteria (LAB) and bifidobacteria, have shown to have beneficial effects on immunomodulation and in the alleviation or prevention of diverse intestinal disorders (Fooks and Gibson, 2002; Perdigón et al., 2002; Servin and Coconnier, 2003). Probiotic bacteria are reported to prevent the adherence, establishment and invasion of specific enteropathogens (Servin, 2004). Several mechanisms have been proposed: contribution to mucosal barrier function, competitive exclusion, modulation of the immune response, coaggregation to pathogens, decreasing of the luminal

* Corresponding author. Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), Calle 47 y 116-La Plata, 1900, Argentina. Tel.: +54 221 4254853; fax: +54 221 4249287.
E-mail address: gdantoni@biol.unlp.edu.ar (G.L. De Antoni).

0168-1605/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2007.07.042

pH via the production of lactic acid and secretion of specific compounds such as bacteriocins (Bernet-Camard et al., 1997; Boris et al., 1998; Coconnier et al., 2000; Moal et al., 2002). Ability to temporarily colonize the intestinal epithelia has been linked with probiotic properties because it will allow probiotic bacteria to exert their beneficial effects longer. (Ouwehand et al., 1999; Servin and Coconnier, 2003).

Several health promoting properties are reported to be associated with kefir consumption. Among the effects reported are prevention of tumors (Murofushi et al., 1983; Hosono et al., 1990) and in the stimulation of the immune system (Osada et al., 1994; Thoreux and Schmucker, 2001; Umeda et al., 2005; Vinderola et al., 2005, 2006). Beneficial effect of kefir can also be attributed to the inhibition of pathogenic microorganims by metabolic products, such as organic acids, secreted by the LAB included in the kefir grain (Garrote et al., 2000). Zacconi et al. (1995) described the role of kefir in the prevention of colonization of chicken by S*almonella*. Recently, the inhibition of the attachment of *Salmonella* to Caco-2 cells by *L. acidophilus* and *L. kefiranofaciens* belonging to kefir microflora was reported (Santos et al., 2003).

Lactobacillus kefir is a lactic acid bacterium isolated from kefir grains or kefir milk (Garrote et al., 2001). It was described that *L. kefir* carries surface layer (S-layer) proteins (Garrote et al., 2004, 2005). S-layers are crystalline arrays composed of (glyco) protein subunits non-covalently linked to each other as well as to the supporting cell wall. S-layer proteins could play a role in the probiotic activity of certain lactobacilli. It was reported that the S-layer protein from *L. crispatus* JCM 5810 is involved in its adhesion (Sillanpää et al., 2000) and is able to inhibit the adhesion of *E. coli* to basement membrane components (Horie et al., 2002). No reports were found about the probiotic activity of *L. kefir* and/or its S-layer and their role in the health promoting properties of kefir. The aim of this work was to evaluate the ability of *L. kefir* strains or their spent culture supernatants to inhibit *S. enteritidis* adhesion to or invasion of Caco-2/TC-7 cells.

2. Materials and methods

2.1. Bacterial strains and growth conditions

L. kefir CIDCA 83113, 83115, 8321, 8325, 8344, 8345, 8347 and 8348 were isolated from kefir grains and identified previously (Garrote et al., 2001; Delfederico et al., 2006). Lactobacilli were grown under aerobic conditions in De Man-Rogosa-Sharpe (MRS) broth (Biokar Diagnostics, Beauvais, France) for 48 h at 30 °C. *S. enteritidis*, isolated from a clinical sample at Hospital de Pediatría Prof. Juan P. Garrahan, Buenos Aires, Argentina, was provided by Dr. H. Lopardo. The identification was performed by conventional biochemical test and serotyping employing somatic and flagelar antibodies. It was grown in nutritive broth (Biokar Diagnostics, Beauvais, France) for 18 h at 37 °C.

2.2. Caco-2/TC-7 cell culture

Caco-2/TC-7 cells which model the mature enterocytes of the small 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% heat-inactivated (30 min, 60 °C) fetal calf serum (GEN SA., Buenos Aires, Argentina), 1% nonessential amino acids (GIBCO BRL Life Technologies Rockville, MD. USA), penicillin (12 UI/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 cell per well in 24-well tissue culture plates (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 postconfluence after 7 days of culture (differentiated cells) at passages between 23 and 30.

2.3. L. kefir spent culture supernatants

L. kefir cultures were centrifuged for 15 min at 10.000 ×*g* and sterilized by filtration through 0.45 μm membrane filter (Millipore Corporation, Milford, MA 01757, USA) to obtain spent culture supernatant. The pH ranged from 4.5 to 4.7. Lactic acid concentration of spent culture supernatant was measured by High Performance Liquid Chromatography. Acid separation was performed with an AMINEX HPX-87H ion exchange column (BioRad Labs, Richmond, CA 94804, USA) and organic acids were detected with an UV detector at 214 nm (Waters ™ 996, Millipore Corporation, Milford, MA 01757, USA). Acid identification was performed by comparing the retention times of the samples with those from standards of several short chain fatty acids (Sigma Chemical Co., St. Louis, MO, USA). Lactic acid concentration in the spent culture supernatants ranged from 51 to 70 mM. Artificially acidified MRS prepared with fresh MRS plus 70 mM DL-lactic acid (Sigma Chemical Co., St. Louis, MO, USA) and adjusted to pH 4.5 served as a control.

2.4. Extraction of S-layer proteins from L. kefir

Lactobacilli were harvested at stationary phase, collected by centrifugation (10000 ×*g* at 10 °C for 10 min), washed twice with phosphate buffered saline (PBS, pH 7.2) and resuspended to ten O.D. units at 550 mn. Washed cells were mixed with 5 M LiCl (J.T. Baker, Mallinckrodt Baker S.A., Edo de Mexico, Mexico) in a proportion of 4 ml of solution per ml of bacterial suspension. The mixture was incubated in a shaking incubator (Environ Shaker, Lab-line Instruments Inc., Melrose Park, IL, USA) at 200 rpm and 37 °C for 60 min to extract non-covalently bound proteins. Then, it was centrifuged (12000 ×*g* at 10 °C for 15 min). The supernatant containing the S-layer protein was dialyzed against bidistilled water for 24 h at 4 °C using a cellulose membrane (SpectraPor membrane tube, MWCO 6000–8000, Spectrum Medical Industries, Rancho Dominguez, California, US). The dialyzed extract was freeze-dried and resuspended in PBS (pH 7.2).

In order to obtain the S-layer proteins from the spent culture supernatant, proteins were precipitated from 50 ml of spent culture supernatants with two volumes of cold acetone (2 h at −20 °C) and resuspended in PBS (pH 7.2). Protein concentration was

determined by the Bradford method (Bradford, 1976). The proteins extracted from bacteria or presents in the spent culture supernatants were analyzed by SDS-PAGE followed by immunoblotting assay with specific anti-S-layer antisera (Garrote et al., 2005).

2.5. Aggregation assays

2.5.1. Autoaggregation

Lactobacilli were harvested at stationary phase, collected by centrifugation (10000 ×*g* for 10 min), washed twice, and resuspended in PBS (pH 7.2). In all experiments, lactobacilli suspension was standardized to $OD_{550}=1.0$ (2×10^8 CFU/ml). Optical density was measured in a spectrophotometer (Spectronic 20D+, Thermo Scientific, Waltham, MA, US) at regular intervals without disturbing the microbial suspension and the kinetic of sedimentation was obtained. Autoaggregation coefficient (AC) was calculated at *t* time according to Kos et al. (2003) as:

$$AC_t = [1 - (OD_t/OD_i)] \times 100$$

where OD_i is the initial optical density at 550 nm of the microbial suspension and OD_t is the optical density at *t* time.

2.5.2. Coaggregation

Lactobacilli suspensions were obtained as described previously. *Salmonella* were harvested in stationary phase by centrifugation during 4 min at 5000 ×*g* and resuspended in PBS (pH 7.2) to obtain the *Salmonella* suspension. One milliliter of *Lactobacillus* suspension (2×10^8 CFU/ml) and 1 ml of *Salmonella* suspension (2×10^8 CFU/ml) were added in glass test tubes. Optical density was measured at regular intervals in order to obtain the kinetic of sedimentation. Coaggregation coefficient (CC) was calculated as stated before but subtracting the corresponding AC at each time.

2.6. Adhesion of L. kefir to Caco-2/TC-7 cells

For the adhesion assay, Caco-2/TC-7 monolayer was incubated with 0.5 ml of lactobacilli suspension (2×10^8 CFU/ml) and 0.5 ml of DMEM for 1 h at 37 °C in a 5% CO_2 — 95% air atmospheres. Then, the monolayer was washed three times with PBS (pH 7.2) and lysed by adding sterile distilled water. To determine the number of viable lactobacilli adhered to Caco-2/TC-7 cells, appropriate dilutions in 0.1% tryptone (Biokar Diagnostics, Beauvais, France) were plated in MRS and colony counts were performed. Adhesion percentage was calculated with the following equation: (Adhered lactobacilli/lactobacilli added to the well)×100.

Experiments were performed in triplicate on three consecutive cell passages.

2.7. Survival of Salmonella in L. kefir spent culture supernatants (SCS)

Salmonella were collected by centrifugation from an 18 h culture (2×10^8 CFU/ml) and the pellet was suspended in the same volume of *L. kefir* spent culture supernatants obtained as described previously. At predetermined intervals, aliquots were removed, serially diluted in 0.1% tryptone and plated on nutritive agar (Biokar Diagnostics, Beauvais, France). Colony counts were performed after incubation at 37 °C for 48 h.

2.8. Salmonella association and invasion assays

Control assays were performed as follows: Caco-2/TC-7 monolayers at post-confluence were washed twice with sterile PBS (pH 7.2) and then 0.5 ml of *Salmonella* suspension (2×10^8 CFU/ml) 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. The monolayers were washed three times with sterile PBS 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.

Salmonella invasion was measured by counting only bacteria located into the Caco-2/TC-7 cells. To perform this experiment the monolayer was washed and incubated with *Salmonella* as described previously. Then, 0.5 ml of gentamicin (100 µg/ml PBS) were added to each well and the monolayer was incubated for 1 h at 37 °C before lysing and colony counts.

Table 1
Surface properties of *L. kefir* strains

Origin (CIDCA grain) [1]	*L. kefir* CIDCA strains [2]	Percentage of adhesion to Caco-2/TC-7 cells [3]	Autoaggregation coefficient at 60 min (AC_t) [4]	Coaggregation coefficient at 60 min (CC_t) [4]
AGK1	83113	2.56±0.70 [a]	0±0 [c]	2.95±0.45 [a]
	83115	0.97±0.30 [a]	51.00±6.00 [a]	13.66±4.80 [b]
AGK2	8321	2.98±1.32 [a]	52.00±5.35 [a]	19.73±8.73 [b]
	8325	1.52±0.58 [a]	57.60±5.50 [a]	5.95±1.20 [c]
AGK4	8344	5.30±0.40 [b]	0±0 [c]	1.92±1.20 [a]
	8345	0.13±0.04 [c]	58.50±5.40 [a]	16.80±2.50 [b]
	8347	1.98±0.35 [a]	29.00±1.00 [b]	5.40±1.20 [c]
	8348	4.50±1.29 [b]	40.00±5.00 [a]	23.10±9.75 [b]

[1]: Different types of kefir grains characterized by their technological and *in vitro* inhibitory properties (Garrote et al., 2000, 2001).
[2]: Different strains were identified and characterized by the use of phenotypic and nucleic acid based methods (Garrote et al., 2001; Delfederico et al., 2006). In all strains, the presence of an S-layer constituted by a polypeptide of 66±1 kDa was described, with the exception of strain 8344 in which a 71±1 kDa polypeptide was observed (Garrote et al., 2004).
[3]: Percentage of adhesion to Caco-2/TC-7 was performed as indicated in materials and methods and calculated as follows:
(adhered lactobacilli / lactobacilli added to the well)×100.
Different letters indicate significant differences at $p<0.05$ by Student's test.
[4]: Autoaggregation coefficient (AC_t) of *L. kefir* strains and coaggregation coefficient (CC_t) of *L. kefir* with *Salmonella* were calculated as follows:

$$AC_t = [1 - (OD_t/OD_i)] \times 100$$

$$CC_t = [1 - (OD_t/OD_i)] \times 100 - AC_t$$

where OD_i was the optical density of the bacterial suspension at 550 nm at $t=0$ min and OD_t was the optical density at 550 nm at $t=60$ min.
[a,b,c]:Different letters indicate significant differences at $p<0.05$ by Student's test.

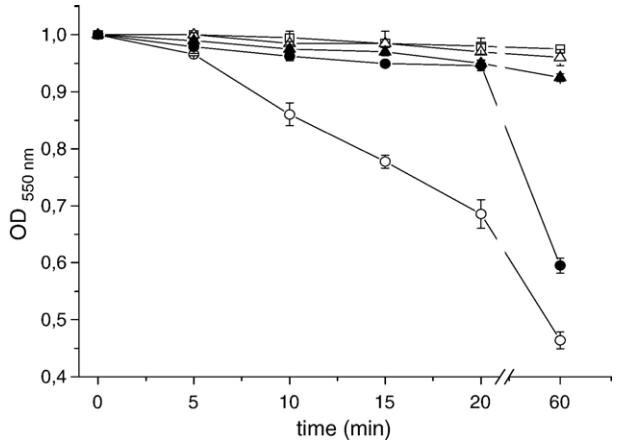

Fig. 1. Autoaggregation kinetics of *Salmonella enteritidis* (□), *L. kefir* CIDCA 8321 (●) and *L. kefir* CDCA 83113 (△). Coaggregation kinetics of *S. enteritidis* with *L. kefir* CIDCA 8321 (○) and with *L. kefir* CIDCA 83113 (▲).

Different types of experiments were performed: i) Caco-2/TC-7 cells were first preincubated with *L. kefir* suspension (2×10^8 CFU/ml) and washed three times with PBS. Then 0.5 ml of *Salmonella* suspension (2×10^8 CFU/ml) and 0.5 ml of DMEM was added to each well. ii) *L. kefir* (2×10^8 CFU/ml) and *Salmonella* (from 4.5×10^7 to 2×10^8 CFU/ml) were mixed and coincubated in PBS (pH 7.2) for 1 h at 37 °C. Then, 0.5 ml of the mixture was added to Caco-2/TC-7 monolayers; iii) *Salmonella* was incubated 1 h at 37 °C with spent culture supernatants at pH 4.5– 4.7 or with S-layer protein resuspended in artificially acidified MRS at different concentrations (0.01, 0.05 and 0.1 mg protein/ml). Then, *Salmonella* pretreated were centrifuged and resuspended in PBS (pH 7.2) and 0.5 ml of *Salmonella* suspension (2×10^8 CFU/ml) was added to each well.

In all cases, *Salmonella* association and invasion assays were performed as described in control assays.

2.9. Dot-blot assay for detection of L. kefir S-layer

One millilitre of *Salmonella* culture was harvested in stationary growth phase and preincubated with an equal volume of spent culture supernatants (pH 4.5–4.7) or S-layer protein from *L. kefir* CIDCA 8321 (0.1 mg/ml in) during 1 hour at 37 °C. Cells were collected by centrifugation (14,000 ×*g*, 10 min at 4 °C) and pellets were washed twice in PBS (pH 7.2), and resuspended in the same buffer. Six microliters of each suspension were placed on nitrocellulose membrane (Micron Separations Inc.; USA).

After coating with *Salmonella* , the membrane was saturated with 3% (w/v) non-fat dry milk dissolved in a buffer containing 0.05 mol/l Tris–HCl, 0.14 mol/l NaCl, 0.0027 mol/l KCl, pH 8.4 (TBS) at 37 °C for 1 h. Then, it was washed three times with TBS plus 0.05% (v/v) Tween 20 (TBS-T) and incubated during 1 h at 37 °C with 1:1000 dilution of specific antiserum against S-layer from *L. kefir* (Garrote et al., 2005).

After washing, peroxidase-labeled second antibody (horseradish peroxidase-conjugated goat anti-rabbit antibody; BioRad Laboratories; Richmond; CA 94804; USA) diluted 1:1000 was applied to the membrane and incubated at 37 °C for 1 h. After another cycle of washing, the reaction was visualized by adding a substrate solution consisting of 9 mg of 4-chloronaphtol

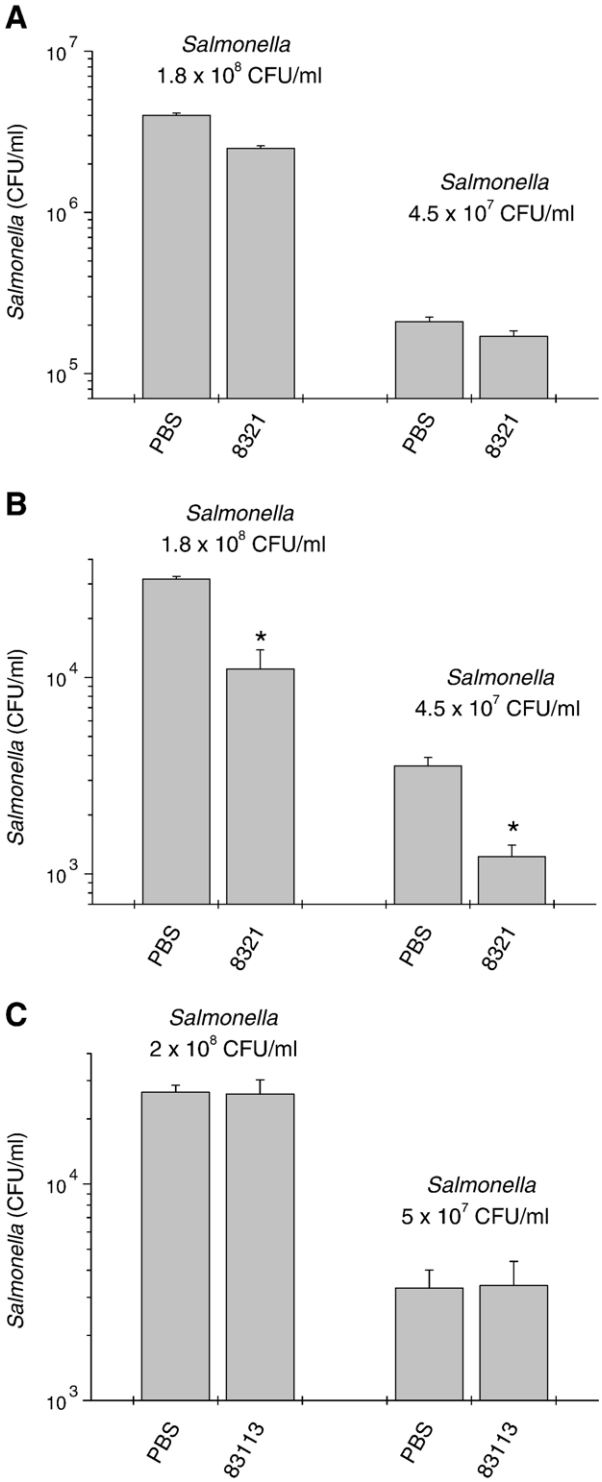

Fig. 2. Association (A) and invasion (B) of *Salmonella enteritidis* coincubated (1 h, 37 °C) with *L. kefir* CIDCA 8321. Invasion of *S. enteritidis* coincubated (1 h, 37 °C) with *L. kefir* CIDCA 83113 (C). Assays were performed with two concentrations of added *Salmonella*. *S. enteritidis* in PBS was used as a control. Error bars indicate standard deviation and * indicates significant differences with corresponding controls at $p<0.05$.

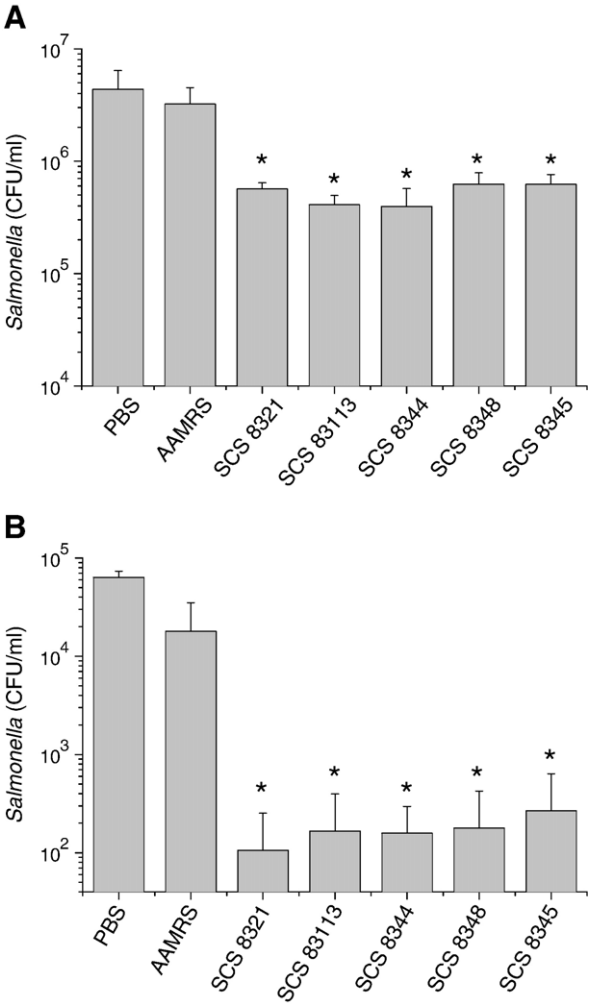

Fig. 3. Association (A) and invasion (B) of *Salmonella enteritidis* pretreated (1 h, 37 °C) with spent culture supernatant of *L. kefir* CIDCA 83113, 8321, 8344, 8345 and 8348. *S. enteritidis* in PBS or pretreated with artificially acidified MRS (AAMRS) were used as controls. In all cases, infection of Caco-2/TC7 was performed at pH 7.2 as indicated in materials and methods. Error bars indicate standard deviation and * indicates significant differences with corresponding controls at $p<0.05$.

(Sigma Chemical Co., St. Louis, MO, USA) and 18 μl of 30% H_2O_2 (E. Merck 64271 Darmstadt, Germany) dissolved in 3 ml of methanol and 15 ml of TBS.

2.10. Scanning electron microscopy

Samples for scanning electron microscopy (SEM) were fixed by incubation in PBS (pH 7.2) containing 2.5% (vol/vol) glutaraldehyde (Riedel de Haen, Seelze, Germany) for 120 min at 4 °C. Then, samples were dehydrated gradually by successive passage through ethanol from 10 to 100%. Samples were placed on isoamylate and dried in a critical-point drying apparatus in liquid CO_2 (Baltec CP-30). Cells were gold coated (Jeol FineCoat Sputter JFC-1100, Jeol Ltd. Akishima Tokyo, Japan) and examined using a Jeol model JSM 6360 LV scanning electron microscope.

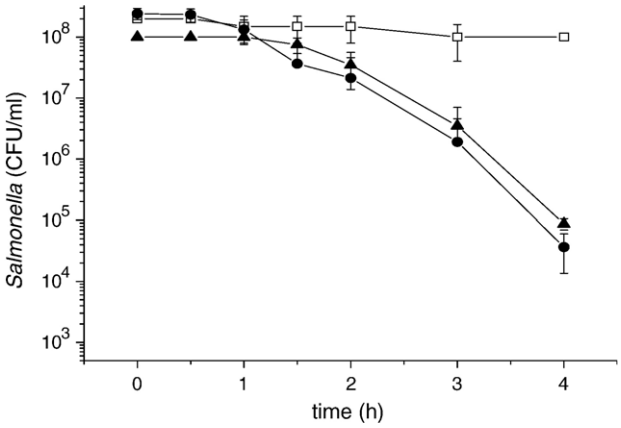

Fig. 4. Decrease in viability of *Salmonella enteritidis* in contact with spent culture supernatants of *L. kefir* 8344 (▲), *L. kefir* 8348 (●) and artificially acidified MRS (□). Error bars indicate standard deviation.

2.11. Data analysis

Results were expressed as means±standard deviation of at least three separate duplicate experiments. For statistical comparisons, Student's *t*-test was performed at *P* value of <0.05.

3. Results

3.1. Surface properties of L. kefir strains

Eight strains of *L. kefir* isolated from different kefir grains were examined for their surface properties (Table 1). Suspensions of *L. kefir* strains with the ability to autoaggregate (e.g. *L. kefir* CIDCA 8321) produced visible clumps and showed a

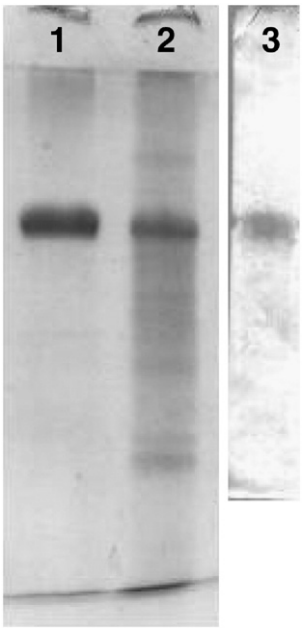

Fig. 5. SDS-PAGE of *Lactobacillus kefir* CIDCA 8344 showing S-layer extracted with LiCl (lane 1) or S-layer precipitated from spent culture supernatant (lane 2). Immunoblotting of surface proteins of *L. kefir* 8344 revealed with specific anti S-layer antisera.

decrease in their OD_{550} not seen in those of non autoaggregating strains (e.g. *L. kefir* CIDCA 83113) (Fig. 1). As shown in Table 1, six strains were able to autoaggregate (CIDCA 83115, 8321, 8325, 8345, 8347 and 8348), and four of these strains (CIDCA 83115, 8321, 8345 and 8348) also showed high coaggregation to *Salmonella*. Bacterial mixtures of *Salmonella* and coaggregating *L. kefir* strains showed a clear difference in sedimentation rate as compared to that of *L. kefir* alone (Fig. 1).

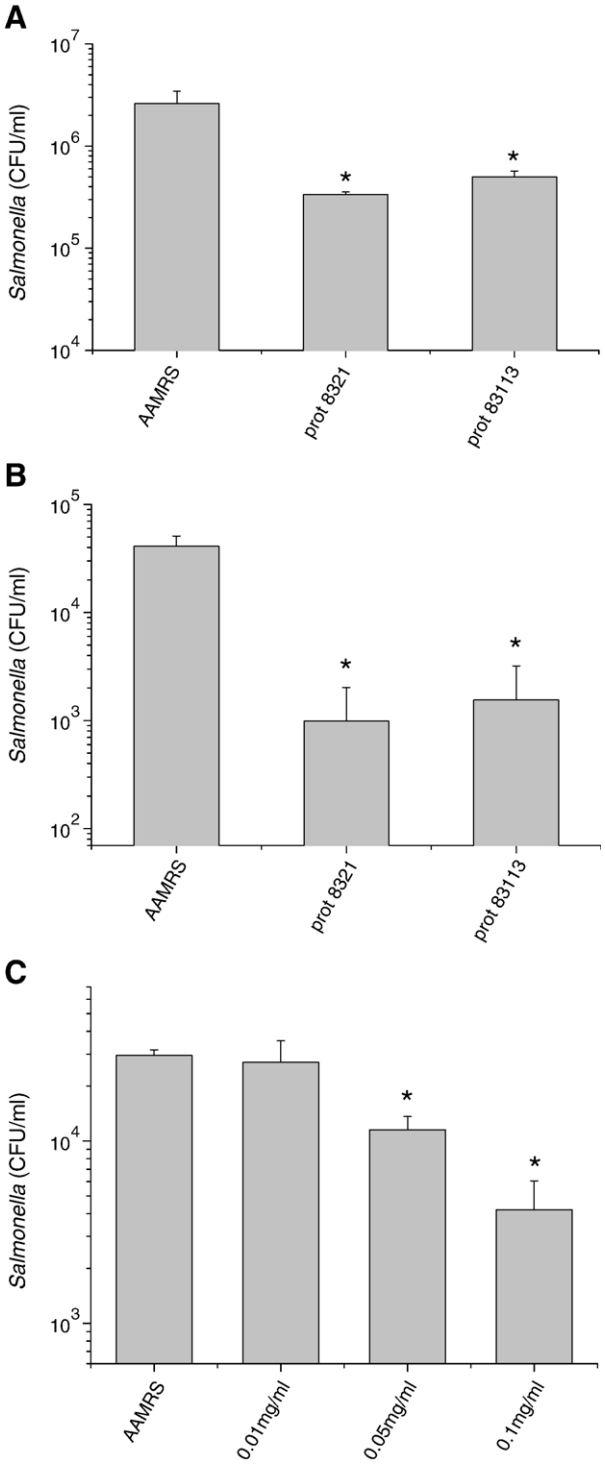

Fig. 6. (A) Association and (B) invasion of *Salmonella enteritidis* pretreated with artificially acidified MRS and 0.1 mg/ml of S-layer extracted from *L. kefir* CIDCA 8321 or *L. kefir* CIDCA 83113. (C) Invasion of Caco-2/TC-7 cells by *S. enteritidis* pretreated with increasing concentrations of S-layer proteins from *L. kefir* CIDCA 8321 in artificially acidified MRS. Artificially acidified MRS without protein was used as a control. Error bars indicate standard deviation and * indicates significant differences with corresponding controls at $p<0.05$.

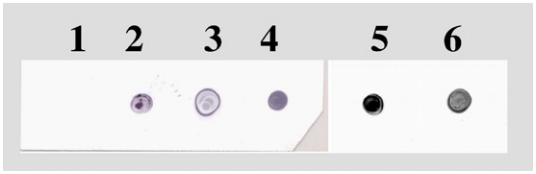

Fig. 7. Dot-Blot assay using a specific antiserum against *L. kefir* S-layer, showing (1) *Salmonella enteritidis*, (2) *L. kefir* CIDCA 8321, (3) spent culture supernatants of *L. kefir* CIDCA 8321, (4) *S. enteritidis* with spent culture supernatants of *L. kefir* CIDCA 8321, (5) *S. enteritidis* with S-layer proteins from *L. kefir* CIDCA 8321, and (6) S-layer proteins from *L. kefir* CIDCA 8321.

L. kefir strains could be clustered in three groups with significantly different abilities to adhere to Caco-2/TC7 cells (Table 1). According to that, one group was represented by CIDCA 8345 strain, the second one by CIDCA 83113, 83115, 8321, 8325, and 8347 strains and the third one by CIDCA 8344 and 8348 strains. The last group showed the same ability to adhere to Caco-2 cells than *Lactobacillus GG* (Kankaanpaa et al., 2001).

3.2. Salmonella association and invasion assays

As different *L. kefir* strains showed different coaggregating abilities (Table 1), we studied the effect of the coincubation of *Salmonella* with a coaggregating (CIDCA 8321) and a non coaggregating (CIDCA 83113) strain on its capacity to associate with Caco-2/TC-7 cells. When *Salmonella* and lactobacilli were coincubated before the infection of a monolayer of Caco-2/TC-7, the results depended on the coaggregative capacity of the *L. kefir* strain. Independently of assayed *Salmonella* concentrations, coincubation with CIDCA 8321 had no effect on *Salmonella* association (Fig. 2A) and clearly protected against *Salmonella* invasion (Fig. 2B). In contrast, CIDCA 83113 did not show any protective action against *Salmonella* association (data not shown) or invasion (Fig. 2C).

On the other hand, when Caco-2/TC-7 monolayers were preincubated with *L. kefir* (2×10^8 CFU/ml) during 1 h at 37 °C, it was found that no one of the strains tested inhibit *Salmonella* association to or invasion of Caco-2/TC-7 cells perhaps due to the low adhesion capacity of *L kefir*.

3.3. Effect of pretreatment of Salmonella with L. kefir spent culture supernatants

Pretreatment of *Salmonella* with spent culture supernatants at pH 4.5–4.7 of *L. kefir* strains CIDCA 83113, 8321, 8344, 8345 and 8348 produced a significant decrease in association (Fig. 3A) and a strong inhibition of invasion (Fig. 3B). Artificially acidified MRS did not show any protective action against *Salmonella* association or invasion (Fig. 3). It is important to remark that *Salmonella* incubated 1 h with spent culture supernatants or artificially acidified MRS did not show any

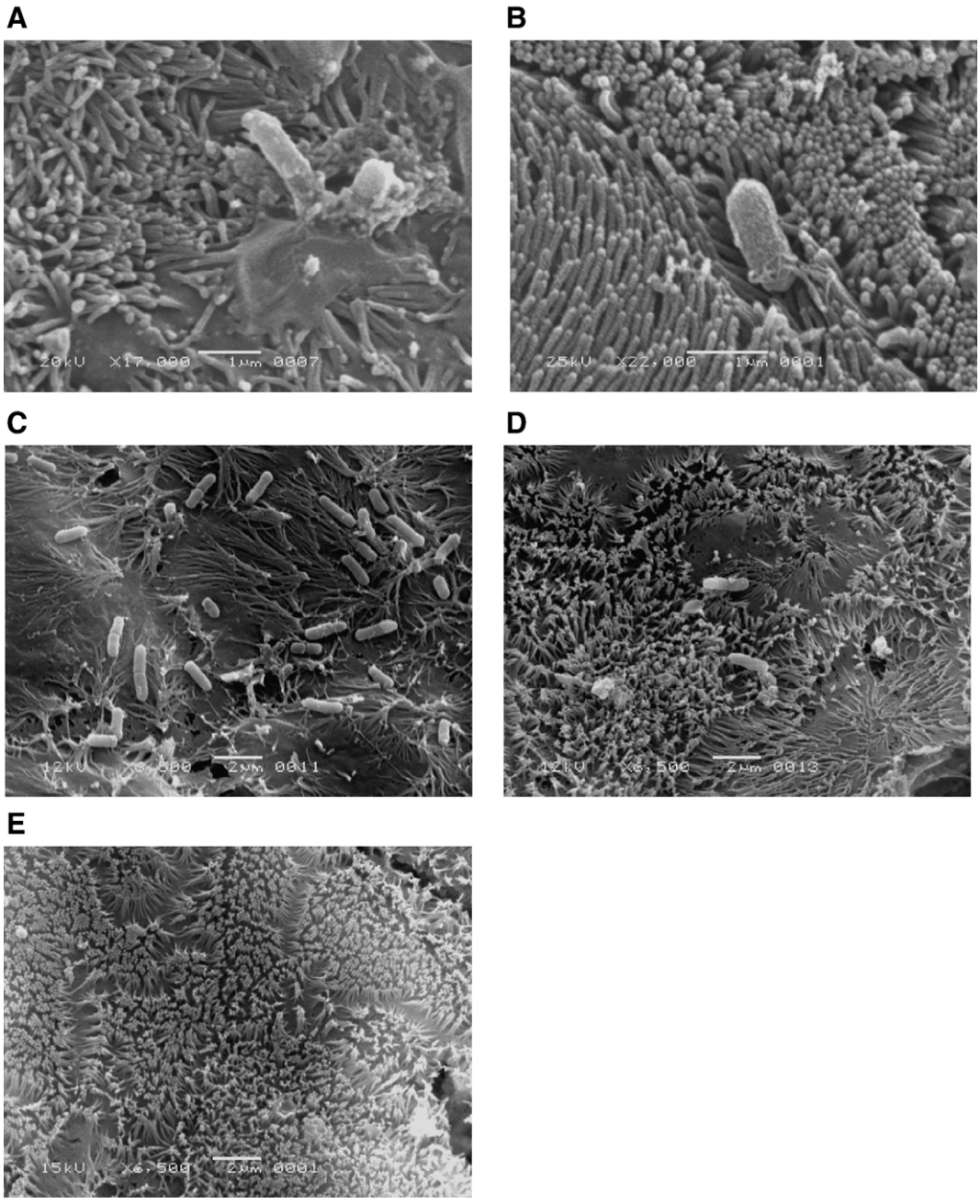

Fig. 8. Scanning electron micrographs of Caco-2/TC7cells infected with *Salmonella enteritidis* during 40 min (A) and 120 min (C). Micrographs of cells infected during 40 min (B) and 120 min (D) with *S. enteritidis* pretreated (1 h at 37 °C) with spent culture supernatants of *L. kefir* CIDCA 8321. Uninfected (E) Caco-2/TC7 cells are also shown.

decrease in viability determined as CFU/ml (Fig. 4). The decrease in *Salmonella* concentration was observed only after 4 h of incubation with spent culture supernatants of CIDCA 8344 and CIDCA 8348 (Fig. 4). The same result was obtained with spent culture supernatants of all studied strains (data not shown).

3.4. Effect of pretreatment of Salmonella with L. kefir S-layer protein

It is known that *L. kefir* carries S-layer proteins on its surface (Garrote et al., 2004). By SDS-PAGE and Immunoblotting assay it was determined that S-layer was the main protein in the spent culture supernatants of *L kefir* (Fig. 5). The estimated concentration of S-layer protein in the spent culture supernatants was 0.1 mg/ml.

Salmonella pretreated with MRS supplemented with 0.1 mg/ml of S-layer extracted from isolates CIDCA 8321 and CIDCA 83113 showed a significant decrease in adhesion to Caco2/TC-7 as compared to the control (Fig. 6A). Contact with 0.1 mg/ml of S-layer proteins from both isolates of *L. kefir* also produced a significant decrease in invasion as compared to the control (Fig. 6B). The protective effect of *L. kefir* S-layer proteins

against *Salmonella* invasion followed a dose-response character (Fig. 6C). Pretreatment of *Salmonella* with *L. kefir* S-layer protein in these experimental conditions did not affect its viability since the incubation of 10^8 CFU/ml of *Salmonella* with S-layer protein did not change their concentration. After pretreatment of *Salmonella* with spent culture supernatants or 0.1 mg/ml of extracted S-layer, the association of this protein with *Salmonella* was detected by Dot-blot (Fig. 7).

To obtain more detailed information about cell structures associated to *S. enteritidis* invasion, time-course infected monolayers of Caco-2/TC-7 were examined by SEM (Fig. 8). Fig 8E shows the intact monolayer of non-infected cells showing intact microvilli over the entire cell surface. At 40 min postinfection, bacterial cells attached and invading the monolayer were detected. In addition, shortened and coalesced microvilli were observed (Fig. 8A). At 120 min postinfection, a higher disorganization of microvilli was observed in *S. enteritidis* infected cells (Fig. 8C). In contrast Caco-2/TC-7 infected with *S. enteritidis* pretreated with spent culture supernatants of *L. kefir* 8321 showed a confluent monolayer containing normal microvilli surrounding *Salmonella* at 40 min postinfection (Fig. 8B). The damage provoked by *Salmonella* pretreated with spent culture supernatant of *L. kefir* was even diminished at 120 min postinfection (Fig. 8D).

4. Discussion

Over the last few years, antagonistic activity of lactic acid bacteria against *Salmonella* infection has been extensively studied (Hudault et al., 1997; Coconnier et al., 2000; Tsai et al., 2005). Although Zacconi et al. (1995) mentioned the antagonistic effect of kefir towards *Salmonella* in chicks and Santos et al. (2003) demonstrated the protective effect of two lactobacilli strains isolated from kefir against *Salmonella*, little is known about the mechanisms implied in inhibition of *Salmonella* by kefir fermented milk.

In vivo, the first direct contact of *Salmonella* ssp with host is the adhesion to the surface of epithelial cells. This event is a prerequisite for the subsequent steps in pathogenesis that lead to mucosal infection, systemic spread and disease (Darwin and Miller, 1999). Inhibition of the invasion of Salmonella into epithelial cells is the first step in disease prevention. Enterocyte-like Caco-2 cells are a suitable system for the study of *Salmonella* adhesion *in vitro* (Pinto et al., 1983; Finlay and Falkow, 1990). In this study, we evaluated the role of *L. kefir* strains isolated from kefir grains against adhesion to and invasion of enterocyte-like cells by *S. enteritidis*. We used the clone TC-7 of Caco-2 cells as an *in vitro* model system to study the effect of *L. kefir* suspensions, their spent culture supernatants and S-layer proteins extracted on *Salmonella* adhesion and invasion. Several authors reported that the inhibition of the attachment of pathogens to Caco-2 cells *in vitro* was directly related with the adhesion capacities of the *Lactobacillus* strains (Fernandez et al., 2003; Lee et al., 2003; Santos et al., 2003). In this work, in contrast, such correlation could not be demonstrated and, moreover, when Caco-2/TC-7 monolayers were incubated with *L. kefir* strains before infection with *S. enteritidis,* adhered lactobacilli did not show any protective action against association and invasion of the enteropathogen. However, we found that significant protection was achieved if lactobacilli and *Salmonella* are previously coincubated. *L. kefir* CIDCA 8321, which co-aggregates with *Salmonella*, has the ability to antagonize *Salmonella* invasion of Caco-2/TC7. In contrast, a non-coaggregating strain (*L. kefir* 83113) does not protect. The above mentioned results suggest that, when coaggregation occurs before the infection of the enterocyte-like cells, a masking of surface structures on *Salmonella* interfere with the invasion process.

The inhibitory effect of lactobacilli spent culture supernatants on the association and invasion of Caco-2/TC-7 cell monolayer by *S. enteritidis* could be associated with extracellular factors present in the spent culture supernatants. Spent culture supernatants contains, among other molecules, organic acids and S-layer proteins. Even though organic acids kill *Salmonella* after a long incubation time (4 h), in the association-invasion assay conditions (1 h of incubation) 99% of the *Salmonella* viability is preserved. Association-invasion assays also show that lactic acid at a concentration equivalent to those obtained in *L. kefir* spent culture supernatants does not inhibit *S. enteritidis* association or invasion. Both experiments confirm that the effect of spent culture supernatants on the inhibition of *Salmonella* association-invasion could not be ascribed to organic acids in the assay conditions.

S-layer proteins have the ability to autoassemble on bacterial surfaces (Garduño et al., 1995; Nomellini et al., 1997; Antikainen et al., 2002). Blotting assays performed with *S. enteritidis* pretreated with S-layer proteins from *L. kefir* strains clearly showed that those proteins remained associated with S*almonella* surface. S-layer proteins could interact with specific sites on *Salmonella* surface involved in the first step of mucosal infection or could either modify or mask *Salmonella* structures necessary for the invasion of cultured human enterocytes. Previously, Horie et al. (2002) reported that not only *L. crispatus* JCM 5810 but also extracted S-layer proteins inhibited the adhesion of *E. coli* to Matrigel, and this effect was ascribed to the competition for the same binding sites in the extracellular matrix. We demonstrated that when *S. enteritidis* is preincubated with *L. kefir* S-layer there is a direct interaction between this protein and *S. enteritidis* surface, instead of a competition for binding sites on the surface of the enterocyte.

We have observed that *L. kefir* CIDCA 83113 is a non coagregating strain that does not interact with *Salmonella* and hence is non protective. However, S-layer proteins isolated from this strain are able to antagonize the *Salmonella* action. This means that, while the association and invasion of *S. enteritidis* to Caco-2/TC-7 cells was not affected by *L. kefir* CIDCA 83113, the internalization of *Salmonella* into the cells was significantly reduced by S-layer protein extracted from this strain. These results could be interpreted taking into account that the conformation or the active groups on the S-layer could change when the protein is in solution in comparison to the protein auto assembled on the *L. kefir* surface. Results obtained in other works demonstrated that S-layer protein is important for the resistance to gastrointestinal passage (Frece et al., 2005), for bacterial adhesion (Schneitz et al., 1993; Hynönen et al., 2002; Antikainen et al., 2002, Frece et al., 2005) and autoaggregation

(Schneitz et al., 1993; Kos et al., 2003). Our results also suggest that preincubation of *Salmonella* with S-layer proteins from *L. kefir* leads to changes in the surface of *Salmonella* thus antagonizing invasion of cultured human enterocytes.

It is known that *Salmonella* interact with microvilli of intestinal brush border and during the entry process, protrusion of the host cell membrane occurs. Changes in the distribution of cytoskeletal component and disorganization of microvilli have been reported during *Salmonella* invasion (Finlay et al., 1989, Finlay and Falkow, 1990). In order to gain information on the effect to *Salmonella* on microvilli structure, infected Caco-2/TC-7 monolayers were examined by SEM. Microvilli of Caco-2/TC-7 cells infected with *S. enteritidis* showed an evident disorganization, whereas a clear protection was achieved when *Salmonella* was pretreated with spent culture supernatants of *L. kefir* CIDCA 8321 which contained S-layer proteins.

The selection of lactobacilli strains with the ability to antagonize *Salmonella* adhesion and invasion could be the first step in the development of a probiotic product to prevent *Salmonella* action in animals and humans. In this work we demonstrated that some strains of *L. kefir* and their S-layer proteins are able to antagonize the adhesion or/and invasion of *S. enteritidis in vitro*.

This work gives further insight on the probiotic properties of lactobacilli carrying S-layer against pathogens. Besides, this is the first report about *L. kefir* S-layer protein interaction with *S. enteritidis* surface, antagonizing adhesion to and invasion of epithelial cells. As *L. kefir* is present in kefir fermented milk, this lactobacilli and their S-layer protein could be partially involved in the probiotic properties of this fermented milk.

Acknowledgements

This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica, Argentina. Financial support from the Consejo Nacional de Investigaciones Científicas y Técnicas, Facultad de Ciencias Exactas (Universidad Nacional de La Plata) and Comisión de Investigaciones Científicas de la Provincia de Buenos Aires are also acknowledged. M. Golowczyc and P. Mobili are fellows of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina). A. Abraham and G. Garrote are researchers of the CONICET and G. L. De Antoni is researcher of the Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC-PBA).

References

Antikainen, J., Anton, L., Sillanpää, J., Korhonen, T.K., 2002. Domains in the S-layer protein CbsA of *Lactobacillus crispatus* involved in adherence to collagens, laminin and lipoteichoic acids and in self-assembly. Molecular Microbiology 46, 381–394.

Bernet-Camard, M.F., Liévin, V., Brassart, D., Neeser, J.R., Servin, A.L., Hudault, S., 1997. The human *Lactobacillus acidophilus* strain LA1 secretes a nonbacteriocin antibacterial substance(s) active *in vitro* and *in vivo*. Applied and Enviromental Microbiology 63, 2747–2753.

Boris, S., Suárez, J.E., Vázquez, F., Barbés, C., 1998. Adherence of human vaginal lactobacilli to vaginal epithelial cells and interaction with uropathogens. Infection and Immunity 66, 1985–1989.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248–254.

Carlson, S.A., Jones, B.D., 1998. Inhibition of Salmonella typhimurium invasion by host cell expression of secreted bacterial invasion proteins. Infection and Immunity 66, 5295–5300.

Coconnier, M.-H., Lievin, V., Lorrot, M., Servin, A.L., 2000. Antagonistic activity of Lactobacillus acidophilus LB against intracellular *Salmonella enterica* serovar Typhimurium infecting human enterocyte-like Caco-2/TC-7 cells. Applied and Environmental Microbiology 66, 1152–1157.

Darwin, K.H., Miller, V.L., 1999. Molecular basis of the interaction of *Salmonella* with the intestinal mucosa. Clinical Microbiology Reviews 12, 405–428.

Delfederico, L., Hollmann, A., Martínez, M., Iglesias, N.G., De Antoni, G., Semoril, L., 2006. Molecular identification and typing of lactobacilli isolated from kefir grains. Journal of Dairy Research 73, 20–27.

Fernandez, M., Boris, S., Barbes, C., 2003. Probiotic properties of human lactobacilli strains to be used in the gastrointestinal tract. Journal of Applied Microbiology 94, 449–455.

Finlay, B.B., Falkow, S., 1990. Salmonella interactions with polarized human intestinal Caco-2 epithelial cells. Journal of Infectious Diseases 162, 1096–1106.

Finlay, B.B., Heffron, F., Falkow, S., 1989. Epithelial cell surfaces induce *Salmonella* proteins required for bacterial adherence and invasion. Science 243 (4893), 940–943.

Fooks, L.J., Gibson, G.R., 2002. *In vitro* investigations of the effect of probiotics and prebiotics on selected human intestinal pathogens. FEMS Microbiology Ecology 39, 67–75.

Frece, J., Kos, B., Svetec, I.K., Zgaga, Z., Mrša, V., Šušković, J., 2005. Importance of S-layer proteins in probiotic activity of *Lactobacillus acidophilus* M92. Journal of Applied Microbiology 98, 285–292.

Garduño, R.A., Phipps, B.M., Kay, W.M., 1995. Physical and functional S-layer reconstitution in *Aeromonas salmonicida*. Journal of Bacteriology 177, 2684–2694.

Garrote, G.L., Abraham, A.G., De Antoni, G.L., 2000. Inhibitory power of kefir: the role of organic acids. Journal of Food Protection 63, 364–369.

Garrote, G.L., Abraham, A.G., De Antoni, G.L., 2001. Chemical and microbiological characterisation of kefir grains. Journal of Dairy Research 68, 639–652.

Garrote, G.L., Delfederico, L., Bibiloni, R., Abraham, A.G., Pérez, P.F., Semorile, L., De Antoni, G.L., 2004. Lactobacilli isolated from kefir grains: evidence of the presence of S-layer proteins. Journal of Dairy Research 71, 222–230.

Garrote, G.L., Serradell, M.A., Abraham, A.G., Añon, M.C., Fossati, C.A., De Antoni, G.L., 2005. Development of an immunochemical method to detect *Lactobacillus kefir*. Food and Agricultural Immunology 16, 221–233.

Horie, M., Ishiyama, A., Fujihira-Ueki, Y., Sillanpaa, J., Korhonen, T.K., Toba, T., 2002. Inhibition of the adherence of *Escherichia coli* strains to basement membrane by *Lactobacillus crispatus* expressing an S-layer. Journal of Applied Microbiology 92, 396–403.

Hosono, A., Tanabe, T., Otani, H., 1990. Binding properties of lactic acid bacteria isolated from kefir milk with mutagenic amino acid pyrolyzates. Milchwissenschaft 45, 647–651.

Hudault, S., Liévin, V., Bernet-Camard, M.-F., Servin, A.L., 1997. Antagonistic activity exerted *in vitro* and *in vivo* by *Lactobacillus casei* (strain GG) against *Salmonella typhimurium* C5 infection. Applied and Environmental Microbiology 63, 513–518.

Hynönen, U., Westerlund-Wikström, B., Palva, A., Korhonen, T.K., 2002. Identification by flagellum display of an epithelial cell- and fibronectin-binding function in the SlpA surface protein of *Lactobacillus brevis*. Journal of Bacteriology 184, 3360–3367.

Kankaanpaa, P.E., Salminen, S.J., Isolauri, E., Lee, Y.K., 2001. The influence of polyunsaturated fatty acids on probiotic growth and adhesion. FEMS Microbiology Letters 194, 149–153.

Kos, B., Šušković, J., Vuković, S., Šimpraga, M., Frece, J., Matošić, S., 2003. Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. Journal of Applied Microbiology 94, 981–987.

Lee, Y-K., Puong, K-Y., Ouwehand, A.C., Salminen, S., 2003. Displacement of bacterial pathogens from mucus and Caco-2 cell surface by lactobacilli. Journal of Medical Microbiology 52, 925–930.

Moal, V.L.-L., Amsellem, R., Servin, A.L., 2002. Lactobacillus acidophilus (strain LB) from the resident adult human gastrointestinal microflora exerts activity against brush border damage promoted by a diarrhoeagenic *Escherichia coli* in human Enterocyte-like cells. Gut 50, 803–811.

Murofushi, M., Shiomi, M., Aibara, K., 1983. Effect of orally administered polysaccharide from kefir grain on delayed-type hypersensivity and tumor growth in mice. Japan Journal of Medical Science Biology 36, 49–53.

Nomellini, J.F., Kupcu, S., Sleytr, U.B., Smit, J., 1997. Factors controlling *in vitro* recrystallization of the *Caulobacter crescentus* paracrystalline S-layer. Journal of Bacteriology 179, 6349–6354.

Osada, K., Nagira, K., Teruya, K., Tachibana, H., Shirahata, S., Murakami, H., 1994. Enhancement of interferon-β production with sphingomyelin from fermented milk. Biotherapy 7, 115–123.

Ouwehand, A.C., Kirjavainen, P.V., Grönlund, M.-M., Isolauri, E., Salminen, S.J., 1999. Adhesion of probiotic micro-organisms to intestinal mucus. International Dairy Journal 9, 623–630.

Patrick, M.E., Adcock, P.M., Gomez, T.M., Altekruse, S.F., Holland, B.H., Tauxe, R.V., Swerdlow, D.L, 2004. *Salmonella enteritidis* infections, United States, 1985–1999. Emerging Infectious Diseases 10, 1–7.

Perdigón, G., Maldonado Galdeano, C., Valdez, J.C., Medici, M., 2002. Interaction of lactic acid bacteria with the gut immune system. European Journal of Clinical Nutrition 56 (SUPPL. 4), S21–S26.

Pinto, M., Robine-Leon, S., Appay, D., Kedinger, M., Triadou, N., Dussaulx, E., Lacroix, B., Simon-Assmann, P., Haffen, K., Fogh, J., Zweibaum, A., 1983. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. Biology of the Cell 47, 323–330.

Santos, A., San Mauro, M., Sanchez, A., Torres, J.M., Marquina, D., 2003. The antimicrobial properties of different strains of *Lactobacillus* spp. isolated from kefir. Systematic and Applied Microbiology 26, 434–437.

Schneitz, C., Nuotio, L., Lounatmaa, K., 1993. Adhesion of *Lactobacillus acidophilus* to avian intestinal epithelial cells mediated by the crystalline bacterial cell surface layer (S-layer). Journal of Applied Bacteriology 74, 290–294.

Servin, A.L., 2004. Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. FEMS Microbiology Reviews 28, 405–440.

Servin, A.L., Coconnier, M.-H., 2003. Adhesion of probiotic strains to the intestinal mucosa and interaction with pathogens. Bailliere's Best Practice and Research in Clinical Gastroenterology 17, 741–754.

Sillanpää, J., Martínez, B., Antikainen, J., Toba, T., Kalkkinen, N., Tankka, S., Lounatmaa, K., Keräen, J., Höök, M., Westerlund-Wikström, B., Pouwels, P.H., Korhonen, T.K., 2000. Characterization of the collagen-binding S-layer protein CbsA of *Lactobacillus crispatus*. Journal of Bacteriology 182, 6440–6450.

Thoreux, K., Schmucker, D.L., 2001. Kefir milk enhances intestinal immunity in young but not old rats. Journal of Nutrition 131, 807–812.

Tsai, C.C., Hsih, H.Y., Chiu, H.H., Lai, Y.Y., Liu, J.H., Yu, B., Tsen, H.Y., 2005. Antagonistic activity against *Salmonella* infection *in vitro* and *in vivo* for two *Lactobacillus* strains from swine and poultry. International Journal of Food Microbiology 102, 185–194.

Umeda, C., Sonoyama, K., Yamaguchi, N., Saito, R., Akashi, K., Motoshima, H., Kawabata, J., 2005. Oral administration of freeze-dried kefir reduces intestinal permeation of and oral sensitization to ovalbumin in mice. Bioscience, Biotechnology and Biochemistry 69, 249–251.

Velge, P., Cloeckaert, A., Barrow, P., 2005. Emergence of *Salmonella* epidemics: the problems related to *Salmonella enterica* serotype Enteritidis and multiple antibiotic resistance in other major serotypes. Veterinary Research 36, 267–288.

Vinderola, C.G., Duarte, J., Thangavel, D., Perdigón, G., Farnworth, E., Matar, C., 2005. Immunomodulating capacity of kefir. Journal of Dairy Research 72, 195–202.

Vinderola, C.G., Perdigón, G., Duarte, J., Thangavel, D., Farnworth, E., Matar, C., 2006. Effects of kefir fractions on innate immunity. Immunobiology 211, 149–156.

Zacconi, C., Parisi, M.G., Sarra, P.G., Dallavalle, P., 1995. Competitive exclusion of Salmonella kedougou in kefir fed chicks. Microbiologie, Aliments, Nutrition 12, 387–390.