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ORIGINAL RESEARCH

Cheese whey fermented with kefir micro-organisms: Antagonism against *Salmonella* and immunomodulatory capacity

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The antagonistic effect against Salmonella *spp. and the immunomodulatory capacity of whey fermented with kefir grains and with three strains isolated from them –* Lactobacillus plantarum *CIDCA 8327,* Lactobacillus kefiri *CIDCA 8348 and* Kluyveromyces marxianus *var.* marxianus *CIDCA 8154 – were evaluated. Both fermented products interfered with the capacity of* Salmonella *spp. to associate and invade Caco-2/TC7 cells and reduced up to the basal level the expression of the CCL20 in response to a pro-inflammatory stimulus on Caco-2 CCL20:luc cells. The products with potential application as probiotics obtained by fermentation of whey with kefir micro-organisms represent an alternative to increase whey economic value.*

Keywords Fermentation, Kefir, Lactic acid bacteria, Yeast, Antagonism, Immunomodulation.

INTRODUCTION

Whey is a co-product of cheesemaking and casein manufacture in the dairy industry. It is a nutrient-rich effluent as its represents almost 90% of the milk volume and retains about 55% of their nutrients, mainly lactose (4.5–5% w/v), soluble proteins (0.6–0.8% w/v) and minerals (8–10% of dried extract) (González Siso 1996). On account of these components, and primarily its proteins and peptides that have high nutritional and functional value, whey has been transformed from a waste to a valuable source of products with applications in food, biotechnology and pharmaceutical industries. Nevertheless, the costs associated to valorisation technologies are not normally tolerable to small and medium factories, for which the development of inexpensive alternative uses of this by-product is of relevance (Prazeres *et al.* 2012).

Kefir is a traditional beverage obtained by fermentation of milk with kefir grains. The grains are gelatinous irregular masses ranging from a few mm to 2 cm of diameter with shape resembling cauliflower inflorescences. They are composed by a wide diversity of lactic acid bacteria (10^7–10^9 CFU/g), acetic acid bacteria (10^5–10^6 CFU/g) and yeasts (10^7–10^8 CFU/g) included in a protein and polysaccharide matrix (Garrote *et al.* 2001). The health promoting properties of kefir have been widely proved (Garrote *et al.* 2010; Guzel-Seydim *et al.* 2011; Ahmed *et al.* 2013).

Kefir grains can be used as starter for whey fermentation as they did not change their acidification capacity, growth rate, macroscopic appearance or chemical and microbiological composition after successive subcultures in this substrate (Londero *et al.* 2012). However, some differences on the microbiological composition of whey and milk fermented with kefir grains have been described, being the concentration of yeasts higher and of lactic acid bacteria lower in the former than in fermented milk (Londero *et al.* 2012). The presence of potentially probiotic micro-organisms (Magalhães *et al.* 2010; Londero *et al.* 2012) and of undissociated organic acids with strong inhibitory activity against *Salmonella enterica* and *Escherichia coli* (Londero *et al.* 2011) has been described for whey fermented with kefir grains.

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The present work attempts to deepen the study of health beneficial properties of whey fermented with kefir grains by analysing its antagonistic effect against *Salmonella* spp. and its immunomodulatory capacity to obtain a low-cost probiotic product to increase whey market value. In addition, the use of strains isolated from kefir as starter for whey fermentation is here explored to obtain a product with defined microbiological composition and more constant quality than by the use of kefir grains. For that purpose, three strains isolated from kefir grains with acid and bile tolerance were selected from 150 strains of the collection of Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA, La Plata, Argentina) on base of their probiotic potential. *Lactobacillus plantarum* CIDCA 8327 was selected for its high adhesion to epithelial Caco-2 cells and its ability to inhibit the growth of *Salmonella enterica* serovar. Typhimurium, *S. enterica* serovar. Gallinarum, *S. enterica* serovar. Enteritidis, *E. coli* and *Shigella sonnei* (Golowczyc *et al.* 2008). *Lactobacillus kefiri* CIDCA 8348 was selected for its high adhesion to intestinal epithelial cells and its ability to co-aggregate with *S.* Enteritidis (Golowczyc *et al.* 2007). This strain also has S-layer that antagonises the cytotoxic effect of *Clostridium difficile* toxins (Carasi *et al.* 2012) and reduced *Salmonella* spp. invasive capacity (Golowczyc *et al.* 2007) on eukaryotic cells *in vitro*. The yeast *Kluyveromyces marxianus* var. *marxianus* CIDCA 8154 was chosen for its capacity to downregulate intestinal epithelial innate response (Romanin *et al.* 2010), adhere to epithelial intestine-derived cells *in vitro* and to survive passage through the gastrointestinal tract of BALB/c mice (Diosma *et al.* 2013). The aim of this work was to evaluate the capacity of whey fermented with kefir grains and with strains isolated from them to antagonise *Salmonella* spp. infection and to downregulate intestinal epithelial innate response *in vitro.*

MATERIAL AND METHODS

Starters

Kefir grains CIDCA AGK10 belonging to Centro de Investigación y Desarrollo en Criotecnología de Alimentos (La Plata, Argentina) collection and *L. plantarum* CIDCA 8327, *L. kefiri* CIDCA 8348 and *K. marxianus* var. *marxianus* CIDCA 8154 isolated from kefir grains and identified in previous works (Garrote *et al.* 2001; Diosma *et al.* 2013) were used as starters. The yeast was cultivated on yeast peptone dextrose broth (yeast extract 10 g/L, peptone 20 g/L, dextrose 20 g/L) and lactobacilli on MRS broth (DIFCO, Detroit, MI, USA) for 24 h at 30 °C. The cells were harvested by centrifugation for 15 min at 10 000 *g* and washed twice by centrifuging with buffer phosphate saline pH 7 (PBS). The concentration of viable micro-organisms was calculated indirectly by measuring the optical density (OD) at 600 nm using curves of OD vs colony-forming units per mL (CFU/mL) previously constructed.

Whey fermentation

Cheese whey powder (Lactogal®, Porto, Portugal) was reconstituted in water (100 g/L) at room temperature with a magnetic stirrer. Flasks containing 100 ml of reconstituted whey were sterilised by Tyndallisation, heating them for 15 min at 100 °C for 3 days in a row. Then, the whey was fermented with kefir grains or strains isolated from them.

Kefir grains CIDCA AGK10 were added to whey at concentration 10% w/v, incubated for 24 h at 20 °C and separated from the fermented whey by filtration through a plastic sieve. *Lactobacillus plantarum* CIDCA 8327, *L. kefiri* CIDCA 8348 and/or *K. marxianus* var. marxianus CIDCA 8154 were inoculated to whey separately or jointly (three strains starter culture) with sufficient quantity of to reach 1×10^6 CFU/mL of each strain and incubated for 72 h at 30 °C.

Enumeration of viable micro-organisms

The concentration of viable micro-organisms in fermented products was determined by plating serial dilutions in tryptone (0.1% w/v) on MRS agar (DIFCO) for lactic acid bacteria (LAB) and on yeast extract–glucose–chloramphenicol (YGC) agar (Merck, Darmstadt, Germany) for yeasts. Results were expressed as CFU/mL in the fermented products.

pH and organic acid determination

The pH of the fermented products was measured with a pH-meter model pH211 equipped with a HI 1330B microelectrode (Hanna Instrument, Ann Arbor, MI, USA). Organic acids were determined both qualitatively and quantitatively by high-performance liquid chromatography as was previously described (Garrote *et al.* 2000).

Antagonistic effect against *Salmonella enterica* serovar. Enteritidis

In vitro growth inhibitory assay

Salmonella enterica serovar. Enteritidis (*S.* Enteritidis) CIDCA 101 was isolated from a clinical sample at Hospital de Pediatría Prof. Juan P. Garrahan, Buenos Aires, Argentina. It was grown in nutrient broth (Biokar Diagnostics, Beauvais, France) for 18 h at 37 °C. Supernatants of whey fermented with kefir grains or with the three strains starter culture were obtained by centrifugation for 10 min at 10 000 *g* and filtration through 0.45-μm membrane filter. They were then diluted in nutrient broth to reach concentrations of 10%, 20%, 30%, 40%, 50% and 60% v/v. Two hundred millilitre of each dilution was inoculated with 5 μL of a *Salmonella* spp. suspension containing 1.5×10^8 CFU/mL, uniformly mixed and incubated at 37 °C. Samples were collected at 30-min intervals, and the OD at 600 nm was measured in a Metrolab 330 spectrophotometer (Buenos Aires, Argentina).

Adhesion and invasion assay to Caco-2 cells

Caco-2/TC-7 cells were routinely cultured according to Golowczyc *et al.* (2007). Cells were seeded at a concentration of 2.5×10^5 cells 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 2 days. Cells were used after 7 days of culture in postconfluence (differentiated cells) at passages between 23 and 30. Caco-2/TC-7 monolayers were washed three times with PBS, and two types of assays were performed.

Treatment of Salmonella *spp. with fermented whey supernatants*

Salmonella enterica serovar. Enteritidis (1×10^6 CFU/mL) was incubated 1 h at 37 °C on supernatants of unfermented whey, whey fermented with kefir grains, whey fermented with the three strains starter culture and whey acidified with lactic and acetic acid to reach the concentrations determined on fermented products. The pH of whey fermented with kefir grains and whey acidified with the same concentration of organic acids was adjusted to 4.5 to avoid the loss of viability of *Salmonella* spp. during incubation. The suspensions were centrifuged, and the pellets were resuspended in Dulbecco modified Eagle's minimal essential medium (DMEM; GIBCO BRL Life Technologies, Rockville, MD, USA). Then, 0.5 mL of each suspension were added to each well and incubated 1 h at 37 °C in a 5% CO_2 – 95% air atmosphere. Caco-2/TC-7 monolayers were washed three times with sterile PBS and lysed by adding sterile water. Appropriate dilutions in 0.1% tryptone were plated on nutrient agar (Biokar Diagnostics), and colony counts were performed to determine the number of *Salmonella* spp. associated (adhering plus invading) to epithelial cells. To evaluate *S.* Enteritidis invasion, 0.5 mL of gentamicin (100 μg/mL PBS) was added to each well and the monolayer was incubated for 1 h at 37 °C to kill bacterial cells attached to enterocytes. Then, the monolayer was washed, lysed and *S.* Enteritidis internalised was counted as described above.

For the evaluation of cellular damage, cells grown on glasses were washed three times with PBS and the F-actin cytoskeleton was labelled with fluorescein-labelled phalloidin (Sigma Chemical Co., St. Louis, MO, USA) as previously described (Minnaard *et al.* 2004). For May-Grünwald/Giemsa stain, the cells were fixed with absolute ethanol for 5 min at 4 °C, washed with PBS and incubated with 0.5 mL of May Grünwald dye solution (Biopur Lab, Argentina) for 3 min. Then, cells were washed with PBS, treated with Giemsa stain diluted 1/10 for 20 min and washed with PBS. Finally, the glasses were mounted on 7 μL of glycerol 50% in PBS with 0.1% sodium azide and observed on a microscope LEICA DMLB (Leica Microsystems, Wetzlar GmbH, Germany) coupled to a DC100 camera (Leica Microscopy Systems Ltd., Heerbrugg, Switzerland).

Treatment of Salmonella *spp. with kefir micro-organisms*

Two millilitres of unfermented whey, whey fermented with kefir grains or whey fermented with the three strains starter culture were centrifuged at 10 000 *g* for 10 min. Pellets were resuspended in 2 or 0.2 mL of PBS. Then, 0.2 mL of these suspensions and 0.2 mL of a *Salmonella* spp. suspension in PBS (4×10^6 CFU/mL) were mixed and co-incubated for 1 h at 37 °C. Then, 0.4 mL of the mixture and 0.4 mL of DMEM were added to each well and incubated 1 h at 37 °C in a 5% CO_2 – 95% air atmosphere.

Caco-2/TC-7 monolayers were treated as described above to determine the number of *Salmonella* spp. associated to or internalised on epithelial cells.

Modulation of CCL20 expression by fermented whey

Whey fermented with kefir grains and whey fermented with the three strains starter culture were centrifuged at 10 000 *g* for 10 min. Pellets were resuspended in PBS and neutralised with 2.5 N NaOH. Confluent Caco-2 CCL20:luc cells cultured in 48-well plates as was previously described for Caco-2/TC7 cells were treated with 125 μL of DMEM and 125 μL of each pellet suspension for 40 min. Cells were stimulated with flagellin (1 μg/mL) for 6 h. Luciferase activity was measured in a Labsystems Luminoskan TL Plus luminometer (Thermo Scientific, Waltham, MA, USA) using a luciferase assay system (Promega, Madison, WI, Waltham, MA, USA) as described by Nempont *et al.* (2008). Luminescence was expressed as a percentage of the mean of untreated stimulated cells (normalised average luminescence, NAL) ± standard deviation (SD) of three independent experiments. The integrity of Caco-2 CCL20:luc cells, grown in 6-well plates, after treatments was evaluated by measuring lactate dehydrogenase (LDH) activity using LDH-P unitest kit (Weiner Lab, Rosario, Argentina) according to the manufacturer's instructions. Results were expressed as the percentage LDH activity in Caco-2 CCL20:luc culture medium in relation to total LDH activity (LDH released after lysing the cells). In both assays, unfermented whey was used as control.

Statistical analysis

Results were expressed as means ± SD of at least three independent triplicate trials. For statistical comparisons, ANOVA and Tukey's test at a 0.05 level of significance were performed.

RESULTS AND DISCUSSION

Whey fermentation by kefir micro-organisms

Whey has been used as a low-cost medium to grown lactic acid bacteria and yeasts to be employed as probiotics (Burns *et al.* 2008) or as starters for dairy industry (Koutinas *et al.* 2009; Vigliengo and Reinheimer 2009). The utilisation of whey would be beneficial in minimising its negative impact

on the environment, as their disposal causes severe contamination resulting in a high biological oxygen demand. To examine the technical feasibility of producing high added value probiotics from whey, it was fermented with kefir grains and with strains isolated from them. In particular, the employment of a defined mixed-starter culture obtained from the traditional kefir grains would be an attractive alternative for commercial implementation. Whey fermented with kefir grains contained $1.0 \pm 0.7 \times 10^7$ CFU/mL of LAB and $4.2 \pm 1.7 \times 10^6$ CFU/mL of yeasts, as was previously reported by Londero *et al.* (2012). LAB growth on whey often required the addition of an extra nitrogen source; however, *L. plantarum* CIDCA 8327 grew in nonsupplemented whey to reach concentrations of 1×10^8 CFU/mL after 72 h of incubation (Figure 1). Instead, *L. kefiri* CIDCA 8348 showed an unsatisfactory growth on whey, with an increase of 1 log order in 72 h. *K. marxianus* var. *marxianus* CIDCA 8154, a lactose fermenting yeast, was able to grow in whey achieving 5×10^7 CFU/mL in 72 h. When the three strains were inoculated simultaneously, *K. marxianus* var. *marxianus* CIDCA 8154 and *L. plantarum* CIDCA 8327 achieved similar viable counts than when they growth in single culture; meanwhile, the growth of *L. kefiri* CIDCA 8348 was enhanced in the mixed culture. *Lactobacillus kefiri* CIDCA 8348 is unable to metabolise lactose; therefore, lactose cleavage by *K. marxianus* and *L. plantarum* could be in part responsible for this performance. A promoting effect of *K. marxianus* var. *marxianus* CIDCA 8154 on the growth of *L. plantarum* CIDCA 83114, a nonlactose fermenting lactobacilli isolated from kefir, has been reported (Echeverría *et al.* 2007). The synergistic effect of *K. marxianus* var. *marxianus* on the growth (Lopitz-Otsoa *et al.* 2006) and the lactic acid production (Plessas *et al.* 2008) of LAB has been also attributed to its production of growth factors such as vitamins.

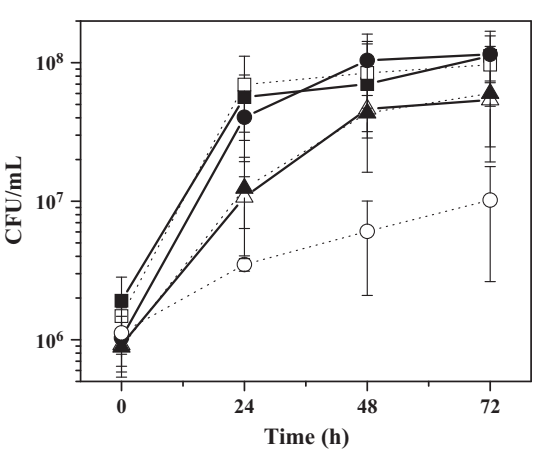

Figure 1 Growth of *Kluyveromyces marxianus* var. *marxianus* CIDCA 8154 (Δ, ▲), *Lactobacillus plantarum* CIDCA 8327 (□, ■) and *Lactobacillus kefiri* CIDCA 8348 (○, ●) inoculated individually (white symbols) or as a three strains starter culture (black symbols) in whey.

The pH and concentration of organic acids in the fermented products were determined (Table 1). Whey fermented with kefir grains had higher lactic acid content despite it has lower micro-organism concentration than whey fermented with the three strains starter culture. That could be due to the organic acid production by the micro-organisms included in the grains matrix which are not released to the medium and therefore are not counted in the fermented product.

The results indicate that nonsupplemented whey is suitable as raw material to obtain two different products by fermentation with kefir grains or the three selected strains in mixed culture. The product obtained by fermentation with kefir strains has the advantage of having a defined microbiological composition leading to products with constant quality.

Antagonistic effect of the fermented products against *Salmonella enterica* serovar. Enteritidis

One of the more widely demonstrated beneficial effects of kefir is it capacity to antagonise intestinal pathogens action (Garrote *et al.* 2010). This effect can be exerted mainly through two mechanisms: direct interactions of the live micro-organisms with pathogens or indirect effects by their metabolites. In the present work, the antimicrobial activity of fermented whey supernatants and the capacity of the microbial and the supernatant fractions to protect the intestinal epithelium against damages caused by *S.* Enteritidis were evaluated.

The growth of *S.* Enteritidis CIDCA 101 in culture medium containing 30% v/v of supernatant of whey fermented with kefir grains was completely inhibited. This result is in accordance with the 30–40% minimum inhibitory concentrations of this product previously reported against 20 strains of *Salmonella enterica* isolated from food samples or infected chickens (Londero *et al.* 2011). The supernatant of whey fermented with the three strains starter culture inhibited the pathogen growth but at concentrations of 50% v/v or higher. The weaker inhibitory capacity of this product in relation to that obtained with kefir grains is in correspondence with its lower undissociated lactic acid content. These results are in agreement with previous works that reports that the inhibitory activity of kefir is largely a result of its high content of organic acids (Garrote *et al.* 2000; Londero *et al.* 2011).

The association and invasion of *Salmonella* spp. to the intestinal epithelium represent a critical step to initiate the infection. The effect of fermented whey supernatants on these processes was evaluated by the use of Caco-2/TC7 intestine-derived cell line. When the pathogen was incubated with the supernatants of whey fermented with kefir grains pH 4.5 or with the three strains starter culture, there were no differences in its association to epithelial cells; nevertheless, a significant decrease on the pathogen invasion was

Table 1 pH and organic acids concentration of whey fermented with kefir grains or with strains isolated from them

Starter	*pH*	*Lactic acid (mM)*	*Acetic acid (mM)*	*Undissociated lactic acid (mM)*	*Undissociated acetic acid (mM)*
Three strains mixed culture[a]	4.5 ± 0.1	51.0 ± 1.1	31.6 ± 3.3	9.5	20.2
Kefir grains[b]	3.6 ± 0.1	99.9 ± 3.3	13.3 ± 0.8	61.9	12.4
None[c]	6.3 ± 0.1	5.5 ± 0.1	2.3 ± 0.5	<0.1	<0.1

[a]Whey was inoculated with *Lactobacillus kefiri* CIDCA 8348, *Lactobacillus plantarum* CIDCA 8327 and *Kluyveromyces marxianus* var. *marxianus* CIDCA 8154 to reach 1×10^6 CFU/mL of each strain and incubated for 72 h at 30 °C.

[b]Whey was inoculated with 10 g/100 mL of kefir grains and incubated for 24 h at 20 °C.

[c]Reconstituted cheese whey powder (Lactogal®) noninoculated was used as control.

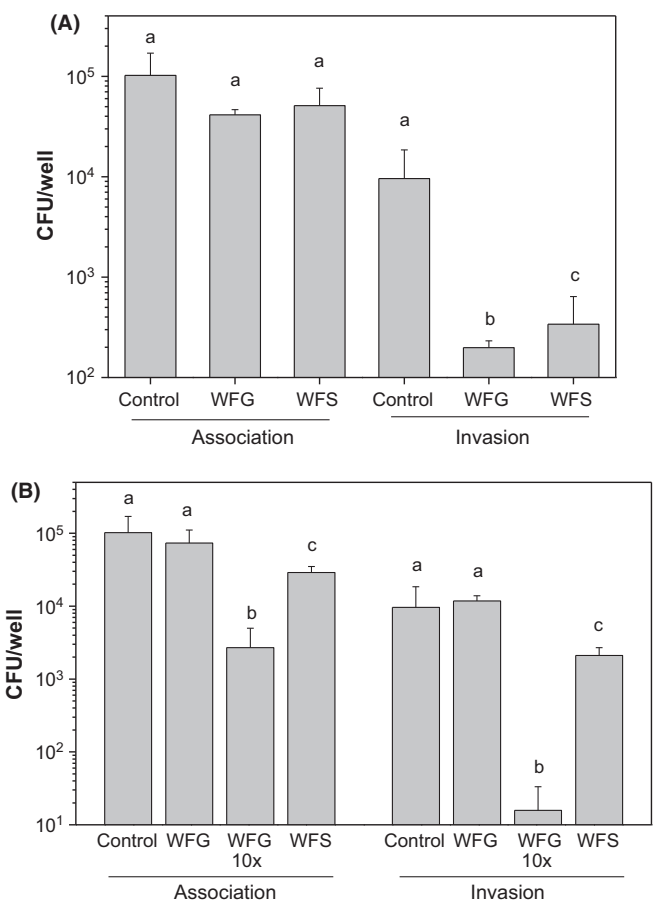

Figure 2 Association and invasion of 1×10^6 CFU/mL of *Salmonella* Enteritidis CIDCA 101 to Caco-2/TC7 cells after being preincubated for 1 h at 37 °C with the supernatant (A) or the micro-organisms (B) of whey fermented with kefir grains (WFG), whey fermented with kefir grains 10 times concentrated (WFG10×) or whey fermented with *Lactobacillus kefiri* CIDCA 8348, *Lactobacillus plantarum* CIDCA 8327 and *Kluyveromyces marxianus* CIDCA 8154 (WFS). *Salmonella* spp. incubated in PBS was used as control. Error bars indicate the standard deviation. Different letters among bars corresponding to *Salmonella* spp. adhesion or invasion indicate significant differences evaluated by ANOVA test ($\alpha = 0.05$).

detected (Figure 2A). This effect was not due to a loss of pathogen viability; therefore, some extracellular factors produced by kefir micro-organisms could interfere with *Salmonella* invasion. When *Salmonella* was incubated in whey acidified with the same concentration of organic acids than fermented products, a similar reduction on the pathogen invasion was detected (data not shown) indicating their implication in the protective effect. Lactic acid has been associated with the reduction of *Salmonella* spp. invasion caused by supernatants of LAB cultures. Hudault *et al.* (1997) described that the invasion of *Salmonella* Typhimurium to Caco-2 cells is reduced when the pathogen is treated with the supernatant of *Lactobacillus casei*. This effect was lost when the supernatant was neutralised, but the pH itself had no inhibitory effect; consequently, the authors stated that the factor responsible for the inhibition is a substance active at low pH produced by the lactobacillus, perhaps lactic acid at a sublethal concentration. Makras *et al.* (2006) proved that culture supernatants of four strains of *Lactobacillus* spp. and a culture medium supplemented with the same concentration of lactic acid exerted identical reduction on *Salmonella* Typhimurium invasion to Caco-2/TC7 cells, indicating that lactic acid is responsible for such inhibitory effect. However, the authors observed that for two of the strains studied (*Lactobacillus johnsonii* La1 and *L. plantarum* ACA-DC 287), the invasion reduction could not be explained only by the presence of this acid, suggesting that another inhibitory substance of unknown nature would be involved. Golowczyc *et al.* (2007) also reported that the protective effect of supernatants from five strains of *L. kefiri* on the invasion of *S.* Enteritidis could not be explained only by its content of lactic acid. These authors showed that spent culture supernatants of *L. kefiri* contain significant amounts of S-layer proteins that remained associated with *Salmonella* spp. surface interfering with its ability to trigger the infection. Results obtained in the present work demonstrate that organic acids present in kefir fermented products are involved in the reduction of *Salmonella* spp. invasiveness capacity, although the possibility of other metabolites could not be omitted.

The effect of the micro-organisms present in fermented products on *Salmonella* spp. capacity to associate and invade intestinal cells was also analysed (Figure 2B). The treatment of *S.* Enteritidis CIDCA 101 (10^6 CFU/mL) with the microbial fraction of whey fermented with kefir grains (10^7 CFU/mL LAB and 10^6 CFU/mL yeasts) did not interfere neither with the association nor the invasion of the pathogen to the enterocytes. Instead, a significant reduction ($P > 0.05$) of these parameters was observed when the analysis was made using the same multiplicity of pathogen infection but 10 times higher kefir micro-organisms concentration (100 LAB and 10 yeasts for each *Salmonella* spp.). A significant decrease in the pathogen association and invasion was also obtained when *Salmonella* spp. was treated with the micro-organisms of whey fermented with the three strains starter culture (10^8 CFU/mL *L. kefiri* CIDCA 8348, 10^8 CFU/mL *L. plantarum* CIDCA 8327 and 10^7 CFU/mL *K. marxianus* var. *marxianus* CIDCA 8154) at the same probiotics/pathogen ratio (Figure 2). Thus, the capacity of the microbial fraction of fermented products to protect Caco-2/TC7 cells against the association and the invasion of *S.* Enteritidis depend on the proportion between the pathogen and the probiotics, being required 100 LAB and 10 yeasts per *Salmonella* spp. to achieve the effect. In accordance with these results, others authors had described that the capacity to protect the intestinal epithelial cells against *Salmonella* spp. invasion is dose dependent (Coconnier *et al.* 1993; Bernet *et al.* 1994).

The microbial fraction of whey fermented with kefir grains had higher protective effect against *Salmonella* spp. association and invasion than the three strains grown in whey when the same relation pathogen/probiotic was evaluated. Whey fermented with kefir grains contains species that were not included in the three strains starter culture, such as *Lactobacillus kefiranofaciens*, *Lactobacillus parakefiri*, *Lactococcus* spp. and *Saccharomyces cerevisiae* (Londero *et al.* 2012), and in kefir, diverse strains of each species with different antagonistic capacity against pathogens coexist (Golowczyc *et al.* 2007; Garrote *et al.* 2010). It is therefore remarkable the protective effect against *Salmonella* spp. achieved by the use of only three kefir strains grown in whey.

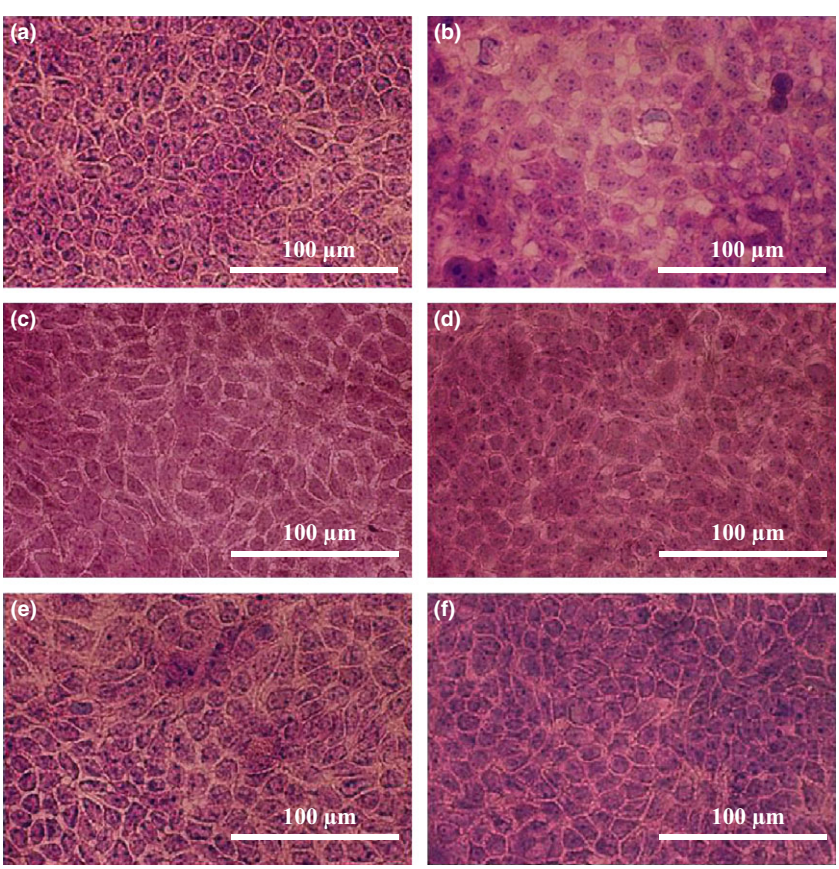

Figure 3 May-Grünwald/Giemsa stained of Caco-2/TC7 cells uninfected (a) or infected with 1×10^6 CFU/mL of *Salmonella* spp. Enteritidis without treatment (b) or pretreated with the supernatant or the micro-organisms of whey fermented with kefir grains (c and d, respectively) or with the supernatant or the micro-organisms of whey fermented with *Lactobacillus kefiri* CIDCA 8348, *Lactobacillus plantarum* CIDCA 8327 and *Kluyveromyces marxianus* var. *marxianus* CIDCA 8154 (e and f, respectively).

Damages caused on Caco-2 cells by *Salmonella* spp. infection were studied by microscopic observation after May-Grünwald/Giemsa staining and phalloidin fluorescein-labelling. Uninfected cells exhibited a uniform appearance with the polyhedral shape typical of this cell line (Figure 3) and the characteristic F-actin distribution on the periphery and the apical surface of the cells (Figure 4). In monolayers infected with *S.* Enteritidis, some cells had a rounded shape and an alteration on F-actin distribution. These changes were not observed when the pathogen was treated previous to the infection with the supernatants or with the micro-organisms of the fermented products (Figures 3 and 4). This reinforces the evidence that these treatments protect the enterocytes from the damage caused by *Salmonella* spp. on the intestinal epithelium.

Downregulation of epithelial innate response by fermented products

The ability of the microbial fraction of fermented whey to modulate the response to pro-inflammatory stimuli on intestinal epithelial cells was analysed employing Caco-2 CCL20 reporter. Chemokine CCL20 is expressed in the gastrointestinal tract under pro-inflammatory conditions such as infections and inflammatory bowel disease (Rumbo *et al.* 2006). The CCL20 promoter is highly inducible in Caco-2 cells upon different pro-inflammatory stimuli being a sensitive indicator of activation of innate response (Nempont *et al.* 2008). When Caco-2 cells were stimulated with flagellin, the expression of CCL20 was increased about 38 times with respect to the basal level. The incubation of the epithelial cells with the microbial fraction of whey fermented with

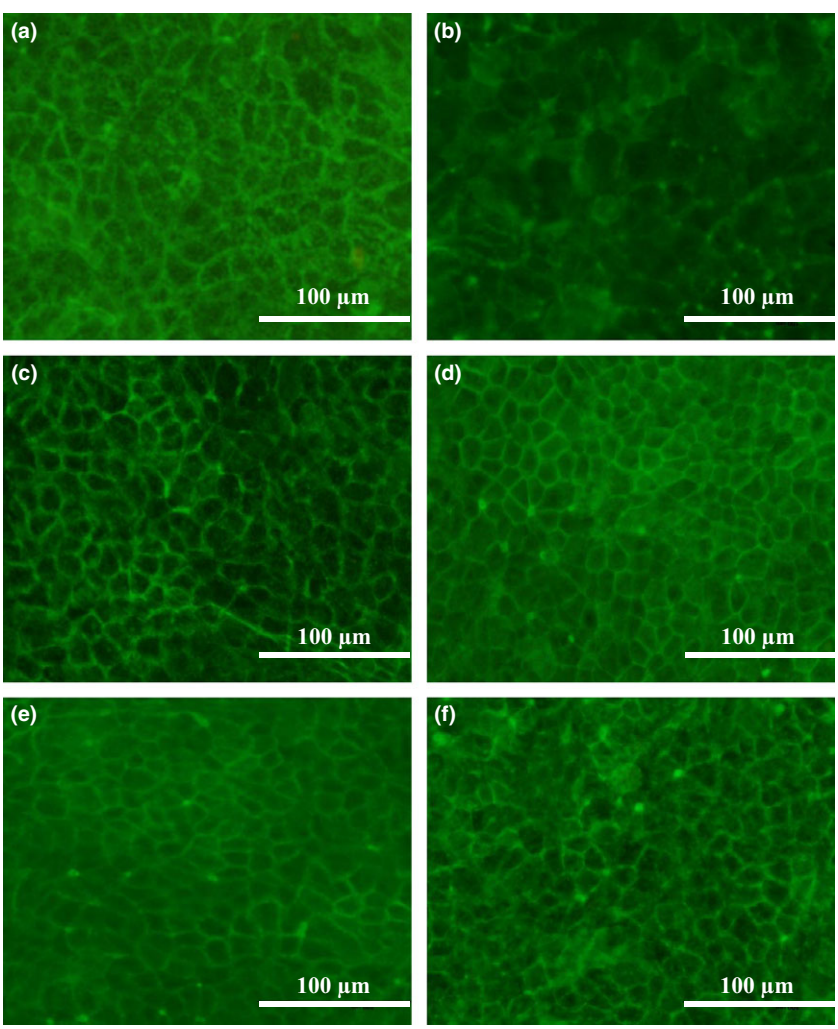

Figure 4 F-actin cytoskeleton labelled with FITC-phalloidin of Caco-2/TC7 cells uninfected (a) or infected with 1×10^6 CFU/mL of *Salmonella* spp. Enteritidis without treatment (b) or pretreated with supernatant or micro-organisms of whey fermented with kefir grains (c and d, respectively) or with supernatant or micro-organisms of whey fermented with *Lactobacillus kefiri* CIDCA 8348, *Lactobacillus plantarum* CIDCA 8327 and *Kluyveromyces marxianus* var. *marxianus* CIDCA 8154 (e and f, respectively).

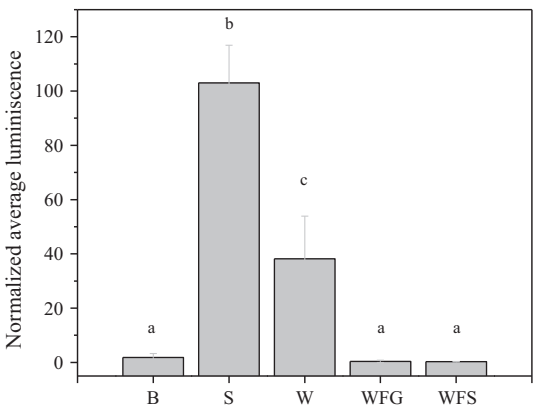

Figure 5 Normalised average luminescence of Caco-2 CCL20:luc reporter cells expressed as percentage of activity induced with flagellin stimulation. Basal activity (B), stimulated with flagellin (S), stimulated with flagellin after being preincubated with pellets of: whey (W), whey fermented with kefir grains (WFG) or whey fermented with *Lactobacillus kefiri* CIDCA 8348, *Lactobacillus plantarum* CIDCA 8327 and *Kluyveromyces marxianus* var. *marxianus* CIDCA 8154 (WFS). Results are expressed as mean ± standard deviation of three independent experiments. Different letters indicate significant differences at $\alpha = 0.05$ (Tukey's test).

kefir grains or with the three strains starter culture reduced the luciferase activity up to the basal level (Figure 5). A 51% inhibition of luciferase activity was observed when Caco-2 CCL20:luc cells were incubated with a pellet suspension of unfermented whey, indicating that an insoluble component of whey is capable to downregulate the expression of the chemokine in response to flagellin, although in lower degree than kefir micro-organisms. The downexpression of CCL20 was not due to a tissue damage as the percentage of LDH activity of all treatments was not significantly different ($P < 0.05$) from the control, with values under 3%.

It has been reported that kefir yeast has high capacity and kefir LAB has moderate capacity to inhibit intestinal epithelial innate response triggered by different pro-inflammatory stimuli *in vitro* (Romanin *et al.* 2010). In the present work, it is demonstrated that the three selected kefir strains grown in whey modulate the inflammatory innate immune response, exerting similar reduction on CCL20 expression than microbial fraction of whey fermented with kefir grains.

CONCLUSIONS

Fermented products with antagonistic activity against *Salmonella* spp. and immunomodulatory capacity were obtained using whey, without any supplement, as a low-cost alternative medium. A product with similar beneficial properties to that obtained by the use of kefir grains was achieved by employing *L. plantarum* CIDCA 8327, *K. marxianus* var. *marxianus* CIDCA 8154 and *L. kefiri* CIDCA 8348 as starter culture, this being a simpler starter, more feasible to be applied for industrial processes.

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