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Development of an immunochemical method to detect *Lactobacillus kefir*

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Abstract

Kefir is a fermented milk elaborated with kefir grains. It constitutes an ecological system composed by a protein-polysaccharide matrix on which a complex microbiota is naturally immobilized. The objective of this work was to develop an antibody-based method in order to achieve a simple and rapid way to quantify *Lactobacillus kefir* in a complex environment. Rabbit antisera against isolated S-layer proteins from *L. kefir* and *L. parakefir* were obtained. Both antisera recognized the 21 strains of *L. kefir* and the three strains of *L. parakefir* analysed by dot-blot. By indirect ELISA, specific antiserum against *L. kefir* S-layer showed reactivity against pure cultures of both *L. kefir* and *L. parakefir,* but did not react with *L. brevis* cells. Competitive ELISA allowed quantification of *L. kefir* at concentrations ranging from 5×10^5 to 10^8 bacteria ml^{-1}, but neither *L. parakefir* nor *L. brevis* pure cultures produced inhibition. Through this approach it was possible to detect 10^8 *L. kefir* ml^{-1} in samples of milk fermented for 48 h at 20°C with different kefir grains. Competitive ELISA developed in our laboratory is a suitable method to detect and quantify *L. kefir* in milk and in consequence it could be applied to analyse the relationship between the composition of microflora and probiotic and technological characteristics of the products.

Keywords: *Kefir milk, lactobacilli, immunodetection, ELISA.*

Introduction

Kefir is a sour fermented milk, sometimes carbonated, with low alcohol content. Kefir grains are clusters of micro-organisms held together by a matrix of polysaccharides and proteins that include primarily lactic acid bacteria (lactobacilli, lactococci, leuconostoc), yeasts and acetic acid bacteria (Angulo et al. 1993, Zourari et al. 1988, Garrote et al. 2001). These micro-organisms are responsible for the lactic, acetic and alcoholic fermentation, which produces a product with particular organoleptic properties. Several health-promoting properties are associated to kefir consumption; in this regard, kefir can be considered as a probiotic product. It has

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been used empirically for the treatment of gastrointestinal and metabolic disorders, atherosclerosis, allergy and tuberculosis (Saloff-Coste 1996). Several studies demonstrated antitumour activity of kefir (Hosono et al. 1990), stimulation of immunity system (Saloff-Coste 1996) and both antibacterial (Zacconi et al. 1995, Ryan et al. 1996, Garrote et al. 2000) and antifungal activity (Saloff-Coste 1996).

On the other hand, kefir-fermented milk presents different technological properties depending on conditions and time of fermentation, and type of grain used (Garrote et al. 1998).

Thus, the interactions between different micro-organisms presented in fermented milk or kefir grains contribute to determine the probiotic and technological properties of the product.

At present, the differential enumeration of the species of lactic acid bacteria present in kefir-fermented products is tedious and time-consuming work. Specifically, the enumeration of *Lactobacillus kefir* or *L. parakefir* in kefir-fermented milk by viable count is almost impossible since the colony morphology of these species is similar to other heterofermentative lactobacilli commonly present in kefir.

The probiotic characterization of kefir-fermented milks and optimization of fermentation conditions requires the enumeration of putative probiotic species like *L. kefir*.

Immunological methods have been used extensively to identify bacteria from a variety of ecosystems. Serological techniques combined with enzyme-linked-immunosorbent assays (ELISA) have been used to distinguish bacteria taxonomically (Ricke et al. 1988), detect physiologically closely related strains and quantify them in mixed cultures (Ricke and Schaefer 1990, Durant et al. 1997).

Several heterofermentative strains of *L. kefir* and *L. parakefir* isolated from kefir grains and kefir-fermented milks have macromolecular paracrystalline arrays that completely cover the bacterial cell surface known as S-layer (Garrote et al. 2004). S-layers are composed of subunits of proteins or glycoproteins and represent 15% of all bacterial proteins. The presence of an S-layer has been reported for several lactic acid bacteria such as *L. acidophilus, L. helveticus, L. casei*, *L. brevis*, *L. crispatus*, *L. fermentum*, *L. bulgaricus*, *L. plantarum* amongst other species (Messner and Sleyter 1992, Moschl et al. 1993, Sara and Sleytr 2000). Since the S-layer is easily extracted from the bacterial surface with chaotropic agents, it was selected as immunogen.

In this work immunochemical assays employing a specific antiserum against S-layer protein were developed to detect and quantify *L. kefir* in kefir-fermented milk.

Materials and methods

Strains and culture conditions

L. kefir CIDCA 8321 and *L. parakefir* CIDCA 8328 (Garrote et al. 2001, 2004) were used to obtain S-layer proteins. Strains used in this study were cultured in MRS broth (Biokar Diagnostics, Beauvais, France) at 30°C for 48 h, under anaerobic conditions.

For dot blot assay the following strains were used: *L. kefir* CIDCA 8310, 8314, 8315, 8317, 8319, 83110, 83111, 83113, 83115, 83116, 8321, 8325, 8326, 8332, 8335, 8343, 8344, 8345, 8347, 8348, *L. parakefir* CIDCA 8322 and 8328, as well as homofermentative lactobacilli CIDCA 8313, 8318, 83114, 8323, 8324, 8329, 8331, 8333, 8337, 8341, 8342 and 8349, isolated from four different kefir grains named

AGK1, AGK2, AGK3 and AGK4 (Garrote et al. 2001). *L. kefir* JCM 5818, *L. parakefir* JCM 8573 and *L. brevis* JCM 1059 were obtained from the Japanese Collection of Micro-organisms (Reiken, Japan). *L. acidophilus* ATCC 314 and *L. helveticus* ATCC 15807 were obtained from American Type Culture Collection (Manassas, VA, USA). Strains of *L. plantarum* 335 and 337 were obtained from Dra. Martino (Martino et al. 1991). Bacterial strains used in this study were cultured in MRS broth (Biokar Diagnostics) at 30°C for 48 h under anaerobic conditions, except *L. acidophilus* ATCC 314 and *L. helveticus* ATCC 15807 that were cultured at 37°C.

Milk fermentation

Kefir grains CIDCA AGK2, washed with sterile distilled water, were inoculated into milk (100 g l^{-1}). After 18 or 48 h of incubation at 20°C, the grains were separated from the fermented milk by filtration.

Enumeration of viable micro-organisms

The concentrations of viable micro-organisms in fermented milk were determined by plating in MRS agar (de Man et al. 1960) serial dilutions of samples in tryptone 1 g l^{-1}. Plates were incubated for 48 h at 30°C under anaerobic conditions. The results were expressed as colony-forming units (cfu) per ml of fermented milk.

S-layer extraction

Bacterial cells were collected by centrifugation at 10,000 × ***g*** for 10 min and washed twice with phosphate buffer, pH 7.0. The pellet was suspended with phosphate buffer to achieve an OD_{600}nm = 10. S-layer was extracted using SDS 2% (w/v) at 37°C with shaking for 60 min and, afterwards, treatment at 100°C for 10 min. Centrifugation at 10,000 × ***g*** for 10 min at 10°C was performed and the supernatant was freeze-dried to concentrate the extracted proteins. These preparations were suspended in urea 8 mol l^{-1} – β × mercaptoethanol 5% (v/v) and analysed by SDS-PAGE electrophoresis.

Electrophoresis

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 10% separating and 5.3% stacking gels using the discontinuous buffer system according to Laemmli (1970). Gels were migrated on a BioRad Mini-Protean II (BioRad Laboratories, Richmond, CA, USA) at 120 volts. Each well was charged with 15 μl (3.0–4.5 μg) of whole surface proteins or purified (electroeluted) S-layer proteins from *L. kefir* CIDCA 8321 and *L. parakefir* CIDCA 8328, respectively. After electrophoresis, gels were fixed and stained Coomassie Blue R-250. As molecular weight (MW) references, phosphorylase B, albumin, ovalbumin, carbonic anhydrase, trypsine inhibitor and α-lactalbumin (94, 67, 43, 30, 20.1, 14.4 kDa, respectively) (Pharmacia Biotech, Sweden) were used.

Isolation of S-layer proteins

S-layer fractions (P21 and P28) were isolated by electroelution from the fractionated proteins by SDS-PAGE. The Electro-Eluter 422 from BioRad was employed. Dialysis

membranes with 12–15 kDa cut-offs were employed. The electroelution was carried out for 4 h using 50 mM ammonium bicarbonate plus 0.1% (w/v) SDS. Ultimately, electroeluted samples were lyophilized.

Antisera production

Two New Zealand white rabbits of 1.0–1.5 kg weight were injected intramuscularly with purified P21 or P28 (2 μg/ml) emulsified with complete Freund's adjuvant. A series of four injections (separated every three weeks) of the same antigen in incomplete Freund's adjuvant was administered. One week after the fourth injection, animals were bled by heart puncture. Both anti-P21 and anti-P28 antisera were filtered and frozen at -80°C.

Immunoblotting assay

Gels contents were electroblotted to 0.45 μm pore size nitrocellulose membranes (Micron Separations Inc., USA) in a Mini Transblot System (BioRad) with transfer buffer containing 0.02 mol l^{-1} Tris-HCl, 0.19 mol l^{-1} glycine, and 20% (v/v) methanol, pH 8.35 at 250 mA for 60 min (Tsang et al. 1983). Free binding sites in the membrane were blocked by incubation with 3% (w/v) non-fat dry milk dissolved in a buffer containing 0.05 mol l^{-1} Tris-HCl, 0.14 mol l^{-1} NaCl, 0.0027 mol l^{-1} KCl, pH 8.4 (TBS) at 37°C for 1 h.

Membrane was sliced vertically, and strips were incubated with appropriate dilutions of anti-P21 or anti-P28 in blocking buffer for 1 h at 37°C. After washing three times with TBS plus 0.05% (v/v) Tween 20 (TBS-T), peroxidase-labelled second antibody (horseradish peroxidase-conjugated goat anti-rabbit antibody; BioRad Laboratories) at appropriate dilution in blocking buffer was applied to each strip for 1 h at 37°C. Blot strips then were washed and incubated with substrate solution consisting of 18 mg of 4-chloronaphthol (Sigma) and 25 μL of 30% H_2O_2 (Merck) dissolved in 6 ml of methanol and 30 ml of TBS.

Dot-blot assay

The bacterial strains were harvested in stationary growth phase and were collected by centrifugation (14,000 $\times \boldsymbol{g}$, 10 min at 4°C). Cell pellets were washed twice in a buffer containing 0.0015 mol l^{-1} KH_2PO_4, 0.0081 mol l^{-1} $Na_2HPO_4 \cdot 2H_2O$, 0.14 mol l^{-1} NaCl, 0.0027 mol l^{-1} KCl, pH 7.4 (PBS), and suspended in the same buffer. Six microlitres of whole lysate were placed on nitrocellulose membrane (Micron Separations Inc., USA).

After sensitization, the membrane was saturated with 3% (w/v) non-fat dry milk dissolved in TBS at 37°C for 1 h. Then, it was washed three times with TBS-T and incubated with three different dilutions (1:100, 1:500 and 1:1000) of anti-P21 or anti-P28 for 1 h at 37°C.

After washing, peroxidase-labelled second antibody (horseradish peroxidase-conjugated goat anti-rabbit antibody; BioRad Laboratories) 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 (SIGMA) and 18 μl of 30% H_2O_2 (Merck) dissolved in 3 ml of methanol and 15 ml TBS.

Indirect ELISA with purified proteins

Polystyrene microtitre plates (NUNC, Maxisorb, Denmark) were coated with the purified proteins (P21 or P28) diluted in PBS buffer at 0.1 μg per well. Incubation was carried out at 4°C overnight. Then wells were washed three times with 200 μl of PBS plus 0.05% (v/v) Tween 20 (PBS-T) and incubated with 3% non-fat dry milk dissolved in PBS at 37°C for 2 h. The plates were washed three times with PBS-T and then 100 μl of dilutions of the respective antiserum ranged from 1:100 to 1:680400 were applied to each well and incubated at 37°C for 1 h. Washing as described above was repeated, and then 100 μl of the peroxidase-labelled second antibody (horseradish peroxidase-conjugated goat anti-rabbit antibody; BioRad Laboratories) diluted 1:4000 was added to each well. The plates were incubated for 1 h at 37°C. After another cycle of washing, the reaction was visualized by adding a solution containing 2 mg ml^{-1} of o-phenylenediamine (Sigma) and 1 $\mu l\, l^{-1}$ of 30% H_2O_2 (Merck) in 0.1 mol l^{-1} citrate-phosphate buffer, pH 5.0. The reaction was stopped by the addition of 50 μl per well of 4N sulphuric acid. The plates were read at 492 nm in an ELISA reader (Tecan microplate reader model SpectraRainbow).

Indirect ELISA with bacterial cell

Reactivity of anti-P21 against *L. kefir* CIDCA 8321, *L. parakefir* CIDCA 8328 and *L. brevis* JCM 1059 was tested. Plates were coated with 100 μl of bacterial suspension at concentration ranging from 5×10^2 cfu ml^{-1} to 5×10^7 cfu ml^{-1} for 30 min at room temperature. After the addition of 100 μl of anti-P21 diluted 1:1000, samples were incubated for 2 h at 37°C. Horseradish peroxidase-labelled second antibody diluted 1:3000 was incubated for 1 h at 37°C. Plates were blocked and washed and the reaction was visualized as previously described.

Competitive ELISA

As a first step, anti-P21 titration was performed by indirect ELISA. *L. kefir* CIDCA 8321 cells at 10^7 cfu ml^{-1} were used to coat the plate. Dilutions of antiserum ranging from 1:100 to 1:1638400 were incubated at 37°C for 2 h. Horseradish peroxidase-labelled second antibody diluted 1:3000 was incubated for 1 h at 37°C. Plates were blocked and washed as previously described. Anti-P21 showed a titre of 1:10000 against *L. kefir* CIDCA 8321. Titre was defined as the antiserum dilution that produced 50% of the maximal absorbance at 492 nm.

For the competitive ELISA, polystyrene microtitre plates (NUNC) were coated with 100 μl per well of a *L. kefir* CIDCA 8321 culture at 10^6,10^7 or 10^8 cfu ml^{-1} and incubated for 30 min at room temperature. Then, plates were centrifuged at $500 \times \boldsymbol{g}$ for 5 min and the supernatant was eliminated. After washing with PBS-T, 200 μl per well of blocking buffer containing 3% (w/v) non-fat dry milk in PBS was added and incubated for 2 h at 37°C. Then wells were washed three times with PBS-T.

Inhibition experiments were performed by pre-incubating equal volumes of 1:10 dilution of fermented milk in PBS (because the high viscosity of the samples) or different concentrations of a *L. kefir* CIDCA 8321 culture (from 10^5 to 7×10^7 cfu ml^{-1}), and anti-P21, both diluted in blocking buffer, for 2 h at 37°C.

The antiserum dilution used was previously determined from the titration curve: 1:5000.

A volume of 100 μl of each of the above mentioned pre-incubated samples was transferred to the respective coated wells for competition with bacteria in solid phase and incubated for 90 min at 37°C. After washing with PBS-T, 100 μl per well of the peroxidase-labelled second antibody (horseradish peroxidase-conjugated goat anti-rabbit antibody; BioRad Laboratories) diluted 1:3000 in blocking buffer was added to each well. The plates were incubated for 1 h at 37°C.

Colour was developed by adding a solution containing 2 mg ml^{-1} o-phenylene-diamine (Sigma) and 1 $\mu l\,l^{-1}$ of 30% H_2O_2 (Merck) in 0.1 mol l^{-1} citrate-phosphate buffer, pH 5.0. The reaction was stopped by the addition of 50 μl per well of 4N sulphuric acid. The plates were read at 492 nm in an ELISA reader (Tecan microplate reader model SpectraRainbow).

Absorbance values were transformed with the logit formula (Tijssen 1985):

$$\text{logit } p = \ln[p/(1-p)], \text{ where:}$$
$$p = Ax/Am$$

using Ax and Am as absorbance values corresponding to the wells with samples or known concentrations of bacteria and wells without competitor, respectively.

Statistical analysis

Differences between the number of viable micro-organisms and ELISA quantification were statistically tested by using Analysis of Variance (ANOVA) to determine any significant difference between methodologies at $p \leq 0.05$. A least square regression was applied in order to evaluate the correlation between both methods.

Results

S-layer protein isolation

S-layer obtained as previously described was isolated by electroelution. To verify the purity of immunogens P21 and P28, a SDS-PAGE was performed with eluted samples. Each sample revealed only one band with MW of 65 kDa in P21 and MW of 69 kDa in P28 (Figure 1A).

Antisera reactivity against surface proteins

Reactivity of rabbit antisera against electroeluted surface proteins from *L. kefir* CIDCA 8321 strain (anti-P21) and *L. parakefir* CIDCA 8328 strain (anti-P28) were tested by immunoblotting and ELISA. Immunoblotting results showed that anti-P21 (diluted 1:2000) and anti-P28 (diluted 1:1000) recognized their specific antigen from whole surface proteins and they did not show reaction with other polypeptides (see Figure 1B, lanes 3 and 4).

Additionally, both antisera were tested against electroeluted proteins P21 and P28 by indirect ELISA (see Figure 2). Although cross reactivity was detected, a difference on titres was observed. The anti-P21 titres were 1:20000 and 1:10000 for P21 and

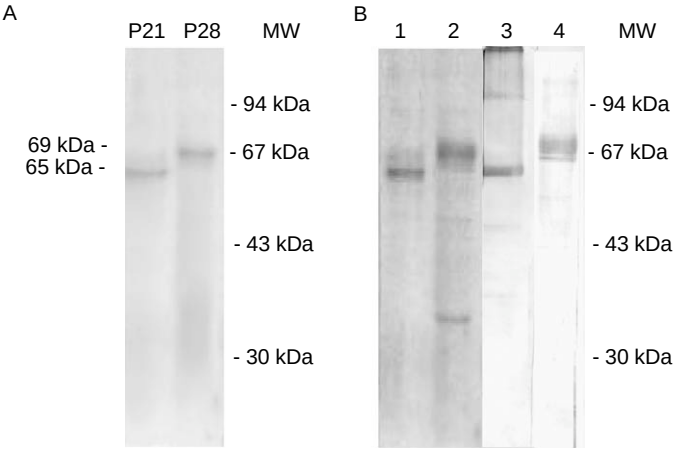

Figure 1. (A) SDS-PAGE of electroeluted S-layer proteins. Lane P21, S-layer from *L. kefir* CIDCA 8321; Lane P28, S-layer from *L. parakefir* CIDCA 8328; MW, molecular weight. (B) SDS-PAGE (lanes 1 and 2) and immunoblotting (lanes 3 and 4) of whole surface proteins. Lane 1, *L. kefir* CIDCA 8321; Lane 2, *L. parakefir* CIDCA 8328; Lane 3, *L. kefir* CIDCA 8321 vs. anti-P21 (dil 1:2000); Lane 4, *L. parakefir* CIDCA 8328 vs. anti-P28 (dil 1:1000); MW, molecular weight.

P28, respectively. On the other hand, anti-P28 titres were 1:10000 for its homologous antigen and $\leq$1:600 for P21 protein.

Antisera reactivity against bacterial cells

Dot-blot assay was performed to test the reactivity of antisera against bacterial surface of different species. Different dilutions of both antisera were capable to detect either

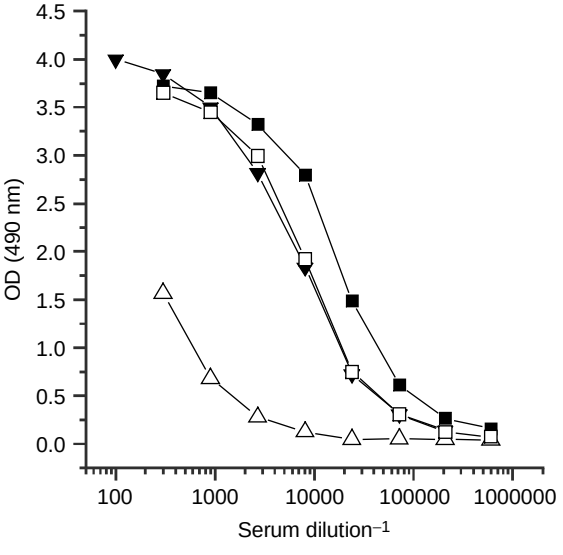

Figure 2. Indirect ELISA of anti-P21 and anti-P28 antisera against both *L. kefir* CIDCA 8321 and *L. parakefir* CIDCA 8328 electroeluted surface proteins. (■) P21 vs. anti-P21; (▼) P28 vs. anti-P28; (□) P28 vs. anti-P21; (△) P21 vs. anti-P28.

the 21 strains of *L. kefir* and the three strains of *L. parakefir* analysed. Neither anti-P21 nor anti-P28 showed reactivity against *L. brevis*, *L. plantarum*, *L. acidophilus*, *L. helveticus*, or all homofermentative lactobacilli isolated from kefir grains. Dot-blot assay performed with anti-P21 antiserum against some the different strains analysed is shown in Figure 3.

Detection of heterofermentative lactobacilli carrying S-layer proteins

Since anti-P21 antiserum showed good reactivity against *L. kefir* and *L. parakefir*, it was selected to develop a method to detect heterofermentative lactobacilli carrying S-layer proteins. Two different immunoassays – indirect and competitive ELISA – were performed.

By indirect ELISA anti-P21 showed reactivity against pure cultures of both *L. kefir* and *L. parakefir* when the concentration was higher than 10^3 cfu ml^{-1}, but did not react with *L. brevis* cells (from 10^2 to 10^7 cfu ml^{-1}) (see Figure 4A). Moreover, a concentration higher than 10^5 bacteria ml^{-1} did not produce an increase of the absorbance reading.

For the competitive ELISA a calibration curve was constructed employing three different concentrations of *L. kefir* CIDCA 8321 bacterial cells in the microplate (10^6, 10^7 y 10^8 cfu ml^{-1}) (Figure 4B). The slopes of the three lineal regressions did not differ significantly, which means that the different concentrations of bacteria used to coat the plate did not modify the response of the assay. Notwithstanding, the sensitization with 10^7 cfu ml^{-1} was selected for quantification assays. Thus, in the chosen conditions it was possible to detect as low as 5×10^5 bacteria ml^{-1} of *L. kefir*. In this method neither *L. parakefir* CIDCA 8328 nor *L. brevis* JCM 1059 pure cultures produced inhibition of the reactivity of antibodies against coating bacterial cells, even at 10^7 cfu ml^{-1}.

Quantification of L. kefir *in fermented milk*

Competitive ELISA was used to detect and quantify *L. kefir* in milk fermented with AGK2 kefir grains. Fermented milk of 48 h of incubation produced inhibition of reactivity of anti-P21 antibodies to *L. kefir* cells. Fermented milk samples of 18 h did

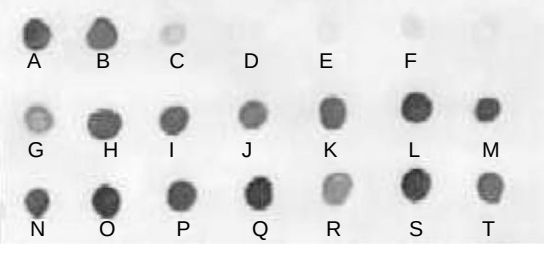

Figure 3. Dot-blot of anti-P21 (diluted 1:1000) against some of the different strains of *L. kefir* (A: CIDCA 8348; B: JCM 5818; H: CIDCA 8332; I: CIDCA 8335; J: CIDCA 8343; K: CIDCA 8344; L: CIDCA 8345; M: CIDCA 8347; N: CIDCA 83113; O: CIDCA 83115; P: CIDCA 83116; Q: CIDCA 8321; S: CIDCA 8325; T: CIDCA 8326), *L. parakefir* (G: CIDCA 8328; R: CIDCA 8322), and strains of micro-organism from other species (C: *L. brevis* JCM 1059; D: *L. plantarum* 335; E: *L. acidophilus* ATCC 314; F: *L. helveticus* ATCC 15807) analysed.

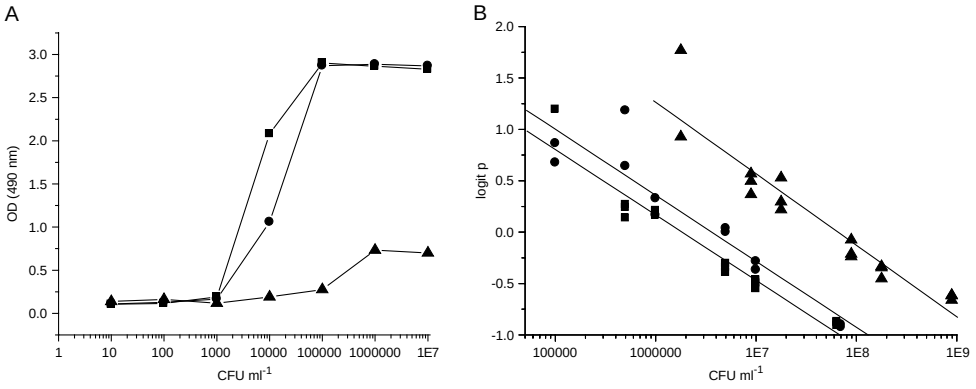

Figure 4. A: Indirect ELISA against *L. kefir* CIDCA 8321 (■), *L. parakefir* CIDCA 8328 (●) and *L. brevis* JCM 1059 (▲) bacterial cells using anti-P21 antiserum. B: Competitive ELISA with anti-P21 against *L. kefir* CIDCA 8321 cells. Different concentrations of *L. kefir* CIDCA 8321 cells were used to coat the microplate: (■) 10^6 cfu ml^{-1}, (●) 10^7 cfu ml^{-1}, (▲) 10^8 cfu ml^{-1}.

not react in these conditions even when these milks contained about 10^7 cfu ml^{-1} of total lactobacilli (see Table I).

In order to verify these results, AGK2 samples, which had been fermented for 18 h, with known concentrations of *L. kefir* CIDCA 8321, *L. parakefir* CIDCA 8328 and *L. brevis* JCM 1059, were tested. Concentration determined by ELISA showed no significant difference ($p > 0.05$) with the values obtained by enumeration of viable bacteria in fermented milk with added *L. kefir* (see Table II). When cultures of *L. parakefir* and *L. brevis* were added to fermented milk, they were not detected by ELISA. This means that the complex microflora present in kefir-fermented milk did not interfere in the ELISA quantification. Additionally, analysis by least square regression showed that the bacterial cell number determined by ELISA increased in proportion to the increase in enumeration by plating with an r^2 value of 0.998.

Additionally, this method was applied to fermented milk with seven different kefir grains (AGK1, AGK2, AGK3, AGK4, AGK5, AGK6 and AGK7) and the results showed that the samples of 48 h of fermentation contained 10^8 *L. kefir* ml^{-1} (see Figure 5).

Table I. Determination of lactobacilli in kefir-fermented milk.

Time of fermentation	Plating in MRS medium (cfu ml^{-1})	Concentration by ELISA (bacteria ml^{-1})
18 h	$(3.4 \pm 0.3) \times 10^7$ ⋆	ND
48 h	$(4.0 \pm 0.4) \times 10^8$ ⋆,†	$(3.8 \pm 0.2) \times 10^8$ ⋆
	$(6.5 \pm 0.5) \times 10^8$ ⋆,†	$(2.0 \pm 2.0) \times 10^8$ ⋆
	$(7.0 \pm 0.4) \times 10^8$ ⋆,†	$(1.8 \pm 0.4) \times 10^8$ ⋆

⋆Values are means of at least two independent experiments ± SD. †Experiments performed with kefir-fermented milks obtained independently. ND: not detected

Table II. Enumeration of heterofermentative lactobacilli in kefir milk of 18 h added with different concentrations of *L. kefir, L. parakefir* or *L. brevis*.

Added strain	Concentration added to milk (cfu ml^{-1})	Concentration by ELISA (bacteria ml^{-1})
L. kefir CIDCA 8321	$(1.8 \pm 0.3) \times 10^8$ ⋆,†	$(4.0 \pm 0.6) \times 10^8$ ⋆,†
	$(1.0 \pm 0.2) \times 10^7$ ⋆,‡	$(1.3 \pm 0.3) \times 10^7$ ⋆,‡
	$(1.0 \pm 0.2) \times 10^6$ ⋆,§	$(2.3 \pm 0.6) \times 10^6$ ⋆,§
L. parakefir CIDCA 8328	$(1.0 \pm 0.3) \times 10^7$ ⋆	ND
	$(1.0 \pm 0.3) \times 10^6$ ⋆	ND
L. brevis JCM 1059	$(1.0 \pm 0.3) \times 10^7$ ⋆	ND
	$(1.0 \pm 0.3) \times 10^6$ ⋆	ND

⋆Values are means of at least two independent experiments ± SD. †,‡,§Values within rows with the same superscript are not significantly different ($p > 0.05$). ND: not detected.

Discussion

Various techniques have been used for the enumeration of specific micro-organisms in samples containing mixed microflora. Traditional plating techniques are time-consuming and the differentiation of individual species using this approach could be imprecise. Particularly, the identification of lactobacilli using these methods usually needs a trained operator.

At present, there is a strong tendency to use molecular methods based in gene amplification to detect micro-organims. In our case, the utilization of S-layer protein gene as a target for a PCR to detect and quantify *L. kefir* in fermented milks would be attractive but we proved (data not shown) that there are several limitations. Cellular disruption is not easy in part because of their envelopes (e.g. S-layer protein) and as a consequence the extraction of DNA is tedious and time-consuming. In addition, obtaining nucleic acid results from a complex matrix such as fermented milk in which other bacteria and yeasts, polysaccharides and milk proteins are present is significantly

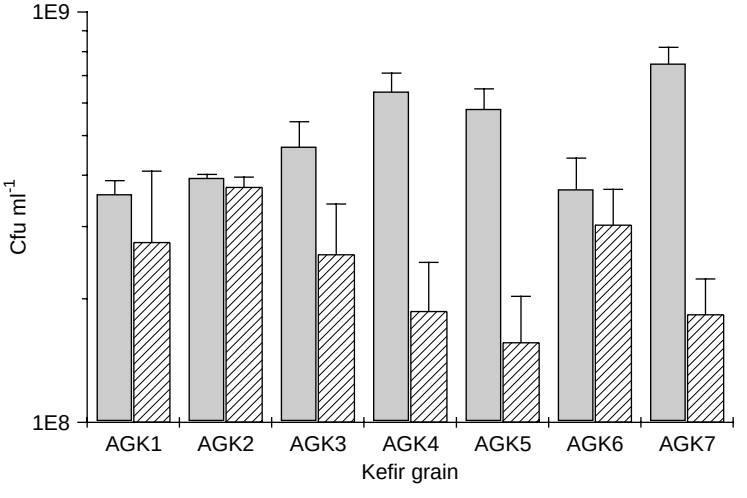

Figure 5. Enumeration of *L. kefir* by competitive ELISA (▨) and total lactobacilli by plating in MRS agar (▬) in 48 h fermented milks obtained with different kefir grains.

more difficult. On the other hand, whilst the sequence of the genes of *L. brevis, L. crispatus, L. acidophilus* and *L. buchneri* S-layer proteins have been described, that of *L. kefir* has not. Hence the use of specific primers is not possible.

Diverse immunological techniques for enumerating specific micro-organisms in mixed microflora have been the subject of extensive investigation and have been applied to different bacterial species. These methods have the advantages of being rapid, specific and sensitive. The selection of an adequate bacterial antigen is a crucial issue to be aware of because it is one of the important factors that can directly affect the specificity of the assay. In the present study, S-layer proteins were used as antigens in order to obtain a polyclonal antiserum and an ELISA assay was developed as an alternative to conventional bacteriological methodology for the rapid detection and quantification of *L. kefir*.

S-layer carrying bacteria could be associated with adherence process to epithelial cells (Schneitz et al. 1993, Toba et al. 1995), thus the detection of S-layer carrying lactobacilli could be a very important fact in order to characterize potentially probiotic kefir-fermented milk. Besides S-layer is the major surface protein and it can be easily extracted from bacterial cell by treatment with chaotropic or denaturing agents. S-layer proteins from different species have been shown different antigenic behaviour. Yasui et al. (1995) reported that rabbit antisera against S-layer of *L. brevis* recognize specifically the homologous strain suggesting S-layer heterogeneity and that this protein could not be used as a species marker for *L. brevis*. In our case, antisera against S-layer of *L. kefir* CIDCA 8321 (anti-P21) recognized by dot-blot all the strains of *L. kefir* analysed and also the S-layer of *L. parakefir*. The antiserum obtained could be used to detect S-layer carrying *L. kefir* and *L. parakefir* isolated from kefir.

Indirect and competitive ELISA developed in our laboratory showed differences in specificity and detection limit. Indirect ELISA detected both *L. kefir* CIDCA 8321 and *L. parakefir* CIDCA 8328 strains; meanwhile competitive ELISA detected only *L. kefir* CIDCA 8321. Although the indirect ELISA seems to be more sensitive than the competitive ELISA in detecting *L. kefir* in a pure culture ($>10^3$ against $>5\times10^5$ bacteria ml^{-1}), it is not a suitable method to be applied when a complex matrix sample is analysed. In the indirect assay, several components of the matrix – other bacteria, yeast, macromolecules, etc – could interfere with the binding of the specific antigen (*L. kefir* cells) to the plate, and consequently affect the sensitivity of the assay. In addition, indirect ELISA does not allow quantification of more than 10^5 bacteria ml^{-1}, meanwhile by competitive ELISA concentrations as high as 10^8 bacteria ml^{-1} can be determined. Taking into account that concentrations of total lactobacilli in kefir-fermented milk are frequently higher than 10^6 cfu ml^{-1} and a complex microflora of yeast and other lactic bacteria is present, the competitive ELISA would be able to detect and quantify *L. kefir* in this kind of products.

Similar immunochemical methods were applied to detect *L. casei* strain Shirota in faeces samples after administration of a fermented product. Yuki et al. (1999) established an indirect ELISA using a monoclonal antibody for identifying colonies after plating on a selective medium. Under the conditions used for ELISA by Yuki et al. about 10^5 bacteria were sufficient for detection, meanwhile indirect ELISA developed in our laboratory is able to detect a lower number of bacteria (10^3 bacteria ml^{-1}).

Besides, our methods do not need a previous selective culture step. This is an advantage because finding an adequate selective culture medium to isolate a specific micro-organism in kefir milk may become a difficult and expensive task.

Fermented kefir milk has a complex microflora that depends principally on time of fermentation, grain-milk ratio and type of grain (Marshall 1993, Garrote et al. 1998). In consequence, the identification and quantification of a particular potentially probiotic micro-organism might be crucial in order to optimize the conditions for the fermentation.

Although total lactobacilli were detected in both 18 and 48 h fermented milk samples by plating in MRS medium, only samples of 48 h of fermentation showed inhibition of binding of anti-P21 to *L. kefir* CIDCA 8321 cells, suggesting that fermentation time could determine the composition of the microflora in the final product.

The antiserum against S-layer of one strain of *L. kefir* isolated from CIDCA AGK2 kefir grain allowed enumeration of *L. kefir* in milk fermented with different grains (see Figure 5). It is important to point out that these grains come from different households, present macroscopic differences in aspect and size, and produce fermented milk with distinct technological properties (Garrote et al. 2001).

In conclusion, the method developed in our laboratory is able to detect and quantify *L. kefir* in kefir-fermented products, which will allow us both to characterize the bacterial microflora and to optimize the conditions for the production of a product with desired probiotic properties.

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