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Interaction between *Lactobacillus kefir* and *Saccharomyces lipolytica* isolated from kefir grains: evidence for lectin-like activity of bacterial surface proteins

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Several microbial interactions involving yeast and lactobacilli have been suggested in fermented products. Co-aggregation between *Lactobacillus kefir* and yeast *Saccharomyces lipolytica* isolated from kefir grains was studied by scanning electron microscopy and aggregation assays. Six out of twenty *Lb. kefir* strains were able to co-aggregate with *Sacch. lipolytica* CIDCA 812 and showed thermolabile non-covalently bound surface molecules involved in this interaction. Co-aggregation inhibition after *Lb. kefir* pre-treatment with 5 M-LiCl or 20 g SDS/l showed that bacterial S-layer proteins play an important role in this interaction. Presence of different sugar (mannose, sucrose and fructose) or yeast pre-treatment with sodium periodate inhibited co-aggregation between *Lb. kefir* and *Sacch. lipolytica*. Co-aggregating *Lb. kefir* strains were also able to agglutinate with human red blood cells and they lost this ability after treatment with 5 M-LiCl. These results and the capacity of purified S-layer proteins of *Lb. kefir* to haemagglutinate, strongly suggest that a lectin-like activity of bacterial surface proteins (S-layer) mediates the aggregation with yeast cells.

Keywords: kefir microorganisms, surface proteins, lectins, co-aggregation.

Kefir is a dairy product obtained by fermentation of milk with kefir grains. Kefir grains are clusters of micro-organisms that include primarily lactic acid bacteria (lactobacilli, lactococci, leuconostoc), yeasts and acetic acid bacteria held together in a matrix of polysaccharides and proteins (Angulo et al. 1993; Garrote et al. 2001). Several health-promoting properties are associated to kefir consumption; indeed, *in vitro* and animal trials showed that kefir and its constituents could have anticarcinogenic, antimutagenic, antiviral and antimicrobial properties (Garrote et al. 2000; Farnworth, 2005).

The interactions between different micro-organisms in kefir grains may contribute to the maintenance of grain structure and composition over time. In this process, ionic or Coulombic interactions, hydrogen bonding, hydrophobic effect or microbial surface macromolecules such as (glyco) proteins and polysaccharides could be involved.

Yeast surfaces have three major cell wall components, namely glucans, mannans, and chitin, all of which may play a role in coaggregation and coadhesion events (Chaffin et al. 1998; Millsap et al. 1998). Mannans form a capsule-like structure on the yeast cell surface and bacteria may associate with sugars in this capsule by means of a lectin-like activity (Millsap et al. 1998).

Our workgroup isolated several S-layer carrying *Lactobacillus kefir* (*Lb. kefir*) strains from kefir grains. These strains showed differences in surface and probiotic properties such as adhesion to Caco-2 cells, bile salts resistance and inhibitory power against intestinal pathogens in vitro assays (Garrote et al. 2001, 2004; Golowczyc et al. 2007).

To gain insight into the nature of the interactions between microorganisms present in kefir grains, we

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characterized surface components of *Lb. kefir* involved in the interaction with *Saccharomyces lipolytica* cells.

Materials and Methods

Strains and media

Yeast and lactobacilli used in this study were isolated from kefir grains (Garrote et al. 2001). *Lactobacillus kefir* strains (CIDCA 8310, 8314, 8315, 8317, 8319, 83110, 83111, 83113, 83115, 83116, 8321, 8325, 8326, 8332, 8335, 8343, 8344, 8345, 8347 and 8348) and *Saccharomyces lipolytica* CIDCA 812 were cultured in MRS broth (Biokar Diagnostics, Beauvais, France) at 30 °C for 48 h.

Surface proteins extraction

Surface protein extraction from *Lb. kefir* with 5 M-LiCl was performed according to Golowczyc et al. (2007). Extraction with SDS was performed using the same experimental procedure with 2% (w/v) SDS (Sigma Chemical Co., USA) in a proportion of 4 ml of solution per ml of bacterial suspension.

Scanning electron microscopy

Samples for scanning electron microscopy (SEM) were collected by centrifugation (6000 ***g*** for 10 min) and fixed by incubation in 2·5% (v/v) glutaraldehyde containing phosphate buffer for 90 min at 4 °C. Then, samples were dehydrated gradually by successive passage through 10 to 100% (v/v) ethanol. Samples were placed on isoamylate and dried in a critical-point drying apparatus in liquid CO_2 (Baltec CP-30). Specimens were sputter coated with gold and examined in a JSM-T 100 (Jeol Ltd, Japón) scanning electron microscope at an accelerating voltage of 20 kV (Bibiloni et al. 2001).

Aggregation assays

Lactobacilli or yeasts were harvested by centrifugation (10 000 ***g*** for 10 min), washed twice and resuspended in 0·5 M-phosphate buffer (pH 7·2). Aggregation assays were performed at 20–25 °C in 0·5 M-phosphate buffer (pH 7·2) unless indicated. In all experiments the initial concentration of microorganisms was standardized to $OD_{550\,nm}$ = 1 approximately. One milliliter lactobacilli suspension and 1 ml yeast suspension were added in glass test tubes. Optical density at 550 nm was measured in a spectrophotometer (Spectronic 20D+, Thermo Scientific, Waltham, MA, US) at regular intervals without disturbing the microbial suspension. Percentage of co-aggregation (% C) was calculated as:

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

where OD_i is the initial optical density of the microbial suspension and OD_t is the optical density at t time (Golowczyc et al. 2007).

In order to identify the structures involved in co-aggregation process, assays were performed after thermal (100 °C in a boiling water bath for 10 min) or protease (2·5 mg ml^{-1} proteinase K, trypsin or chymotrypsin, obtained from Sigma Chemical Co., in 0·05 M-Tris-HCl buffer, 0·1 M-NaCl, pH 8, at 37 °C for 1 h, followed by enzyme inactivation with bovine fetal serum and washing) bacterial treatments. Co-aggregation assays were also performed after the extraction of S-layer (as indicated above) or in presence of 0·024 mg ml^{-1} S-layer protein from *Lb. kefir*.

To study the nature of these interactions, co-aggregation assays were performed in 0·5 M-phosphate buffer at different pHs (3 or 10), or in 0·5 M-phosphate buffer (pH 7·2) added with 0·1 M-sugar (mannose, fructose, sucrose or glucose, obtained from Sigma) or after yeast treatment with 0·05 M-sodium periodate at 37 °C for 30 min.

Haemagglutination assay

Haemagglutination activity was studied in U-bottom microtitre plates (Nunc, Roskilde, Denmark). Bacterial cells were washed twice with 0·5 M-phosphate buffer and resuspended in the same buffer to an $OD_{550\,nm} = 3$. Twenty-five microliters of two-fold serial dilutions of bacterial suspensions were mixed with 25 µl 2% human erythrocytes suspension. After incubation for 1 h at room temperature, the haemagglutination was observed and confirmed by microscopic observation of the erythrocyte clusters stabilized by bacterial bridges (Bibiloni et al. 2001).

Statistical analysis

Differences in percentage of co-aggregation were statistically tested by using Analysis of Variance (ANOVA) to determine any significant difference between effects and/or interactions at $P \leqslant 0{\cdot}05$ (SYSTAT software, version 2.1).

Results

Lactobacilli-Yeast interaction

Interaction between *Lb. kefir* and *Sacch. lipolytica* CIDCA 812 isolated from kefir grains was studied and percentages of co-aggregation (% C) of 20 different lactobacilli strains with the yeast were calculated. Only six *Lb. kefir* strains had the ability to co-aggregate with the yeast under study (Table 1). As an example, Fig 1 shows the microscopic aspect of a coaggregating strain and a non coaggregating one. A tight surface interaction between *Lb. kefir* CIDCA 8321 and *Sacch. lipolytica* CIDCA 812 was observed by scanning electron microscopy (Fig 1A). Optical microscopy showed bigger clumps in co-aggregating mixtures than in non-co-aggregating ones (Fig 1B & C).

In standardized conditions microbial co-aggregating mixtures showed a high percentage of aggregation (Fig 2). It is important to point out that co-aggregation takes place

Table 1. Co-aggregation of *Lb. kefir* with *Sacch. lipolytica*

Values are means ± SD for at least $n=3$

Strain CIDCA	Percentage co-aggregation at t=5 min
83115	33·3 ± 1·4
8321	34·5 ± 3·7
8325	27·9 ± 6·0
8345	42·7 ± 6·1
8347	30·1 ± 2·3
8348	28·1 ± 2·4
8310-8314-8315-8317-8319	0
83110-83111-83113-83116	
8326-8332-8335-8343-8344	

in a short time in which no aggregation of individual microorganisms is observed.

Role of bacterial surface proteins in lactobacilli-yeast interaction

Co-aggregation of *Lb. kefir* with *Sacch. lipolytica* was studied before and after different treatments. Heating of bacterial cells at 100 °C for 10 min completely inhibited the co-aggregation, while treatment of strains CIDCA 8321 or CIDCA 8347 with three proteolytic enzymes and changes in pH had no effect on co-aggregation (Table 2).

Treatment of *Lb. kefir* with LiCl or SDS completely eliminates S-layer protein from bacterial cells (Garrote et al. 2004) and abolished their co-aggregation ability (Table 2). In addition, the co-aggregation of yeast CIDCA 812 with *Lb. kefir* CIDCA 8321 was diminished when the assay was performed in the presence of extracted *Lb. kefir* surface proteins (S-layer), whereas the auto-aggregation of the yeast was increased when these proteins were present (Fig 3). Note again that no significant auto-aggregation of the yeast occurs in the period in which co-aggregation takes place.

Evidence for lectin-like activity of bacterial surface protein

In order to demonstrate a lectin-like activity on the surface of lactobacilli, co-aggregation assays were performed after yeast surface carbohydrate oxidation with periodate or in presence of different sugars (Table 3). The pre-treatment of yeast CIDCA 812 with sodium periodate completely eliminated the co-aggregation with all *Lb. kefir* studied. The percentage of co-aggregation for strains *Lb. kefir* CIDCA 8321, 8325, 8345, 8347 y 8348 was significantly lower than control when the assay was performed in the presence of mannose. Presence of fructose inhibited significantly the co-aggregation of *Lb. kefir* CIDCA 83115, 8321, 8347, whereas the presence of sucrose affected *Lb. kefir* CIDCA 8321, 8325, 8345 and 8348 co-aggregation. In contrast, glucose had no effect in the co-aggregation on all lactobacilli strains studied (Table 3).

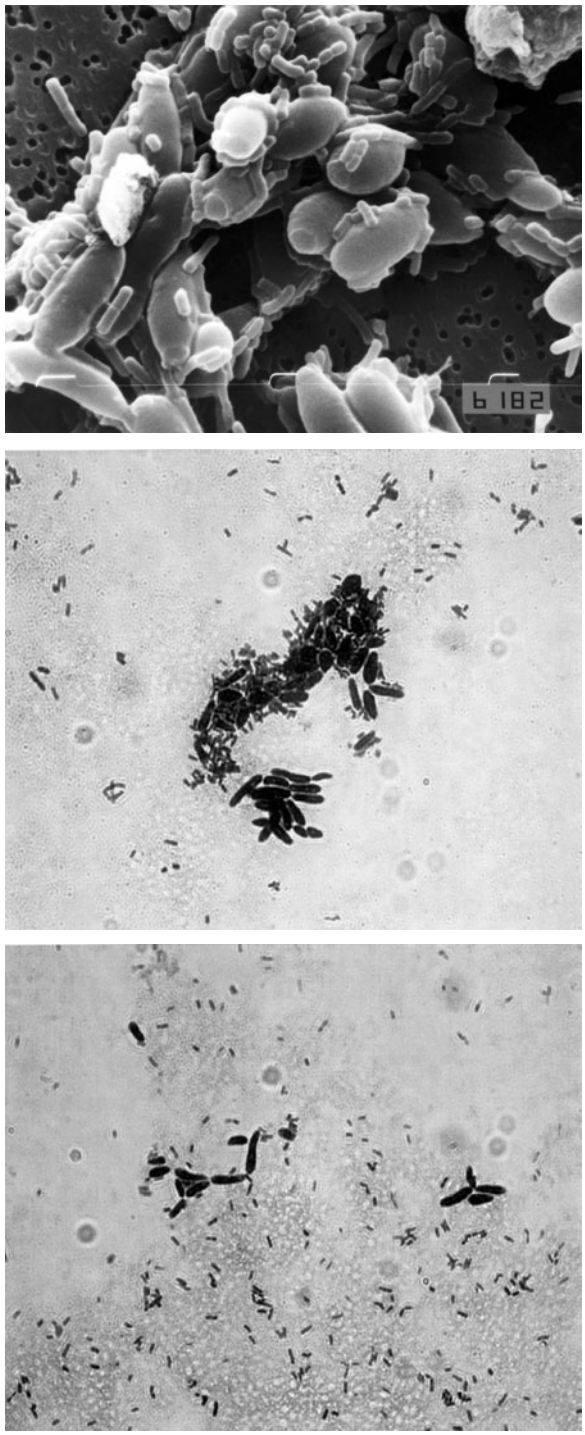

Fig. 1. Scanning electron micrographs of *Lb. kefir* CIDCA 8321 and *Sacch. lipolytica* CIDCA 812 (5000×) (A). Optical microscopy (Gram staining) of the same suspension (1000×) (B). Optical microscopy (Gram staining) of *Lb. kefir* CIDCA 83113 and *Sacch. lipolytica* CIDCA 812 (C).

Haemagglutination assay showed that all aggregative *Lb. kefir* strains studied in this work had the ability to agglutinate with human red blood cells expressing different

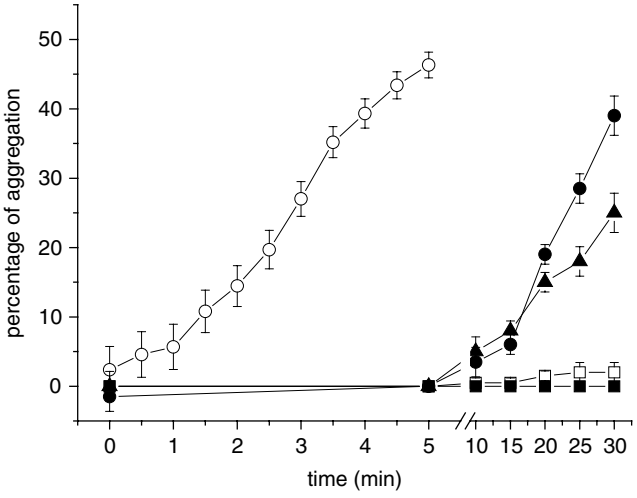

Fig. 2. Co-aggregation assays of *Sacch. lipolytica* CIDCA 812 with *Lb. kefir* CIDCA 8321(○) or CIDCA 83113 (□). Auto-aggregation assays of *Lb. kefir* CIDCA 8321 (●), *Lb. kefir* CIDCA 83113 (■), *Sacch. lipolytica* CIDCA 812 (▲) are also shown.

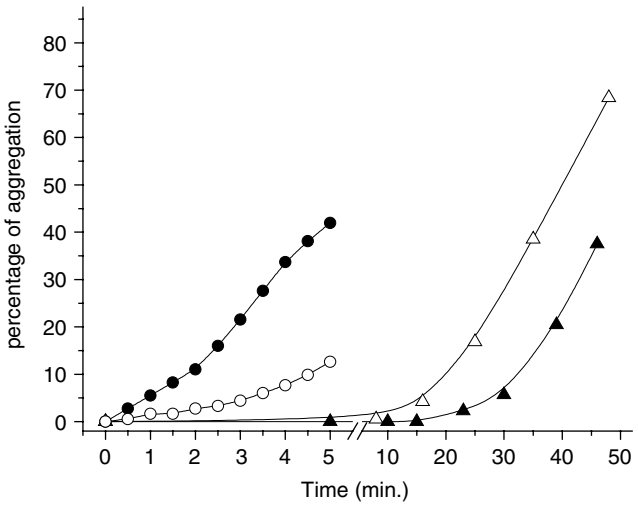

Fig. 3. Aggregation assays in 0·5 M-phosphate buffer (pH 7·2) (solid symbols) and in the same buffer with 0·024 mg ml^{-1} surface proteins from *Lb. kefir* 8321 (open symbols). Co-aggregation of *Sacch. lipolytica* CIDCA 812 and *Lb. kefir* CIDCA 8321 (●, ○) and auto-aggregation of *Sacch. lipolytica* CIDCA 812 (▲, △).

Table 2. Influence of different pretreatments on *Lb. kefir*–*Sacch. lipolytica* interaction

Values are means ± SD for at least $n=3$

	Percentage co-aggregation	
Pretreatment	CIDCA 8321	CIDCA 8347
None (control)	34·5±3·7	30·1±2·3
pH=3	34·5±11·0	30·5±10·5
pH=10	41·3±6·3	30·1±2·15
100 °C, 10 min	0*	0*
trypsin	33·6±4·2	29·3±3·2
chymotripsin	35·1±5·3	32·8±4·3
proteinase-K	44·5±5·0	38·7±2·0
SDS	0*	0*
LiCl	0*	0*

* Significantly different from the control ($P<0·05$)

ABO antigens (Table 3). In addition, this ability was lost after treatment with LiCl and purified S-layer proteins were able to induce the agglutination of human red blood cells. As example, Fig 4 shows the results obtained with *Lb. kefir* CIDCA 8321. In addition, it is important to point out that purified S layer is able to induce haemaglutination. (Fig 4, line C dilution 1/2)

Discussion

Co-aggregation is a process by which genetically distinct microorganisms become attached one to another via specific molecules. Cumulative evidence suggests that such adhesion influences the development of complex multi-species biofilms (Nikolaev & Plakunov, 2007). Kefir grains constitute a natural complex ecosystem in which different microorganims, such as lactic and acetic acid bacteria and yeast, are naturally immobilized into a protein-polysaccharide matrix. Adhesion of microorganims to the matrix and co-aggregation between them could have an important role in the maintenance of the number and species balance in the kefir grain over time. The microorganims attached into the grain probably have advantages over free living microorganisms in reference to the survival in stress conditions such as low pH, low nutrient concentration and sub-optimal temperatures.

In the present work we demonstrate that there is a strong surface interaction between *Lb. kefir* and *Sacch. lipolytica* isolated from kefir grains. This interaction is strain-specific since only six out of 20 *Lb. kefir* strains had the ability to co-aggregate with the selected yeast. Strain specificity was also previously reported by Pretzer et al. (2005) for *Lb. plantarum* strains with different capacity to agglutinate with *Sacch. cerevisiae*.

Inhibition of co-aggregation after heating of bacteria showed that surface molecules involved in the interaction of *Lb. kefir* and *Sacch. lipolytica* CIDCA 812 are thermolabile, suggesting that proteins act as mediators in the aggregation process.

Garrote et al. (2004) described that strains of *Lb. kefir* isolated from kefir present S-layer, a macromolecular paracrystalline array of proteins that completely cover bacterial cell surface. It has been described that, in lactobacilli, S-layer participates in adherence to extracellular matrix components (Antikainen et al. 2002; Avall-Jääskeläinen & Palva, 2005; De Leeuw et al. 2006; Jakava-Viljanen & Palva, 2007) and in interactions with other microorganims

Table 3. Effect of periodate and soluble sugars in the interaction of *Lb. kefir* with *Sacch. lipolytica* (co-aggregation) and ability of *Lb. kefir t*o agglutinate red blood cells (haemagglutination)

	Co-aggregation with *Sacch. lipolytica* CIDCA 812						Haemagglutination			
			Soluble sugars (0·1 M)							
Strains CIDCA	Control	Sodium periodate (0·05 M)	Mannose	Fructose	Sucrose	Glucose	A	B	AB	0
83115	100±6·54	0	92·83±10·59	29·28±11·21*	102·18±11·21	102·18±10·59	–	+	+	+
8321	100±10·66	0	68·98±8·69*	36·52±13·04*	62·60±10·14*	136·20±22·6	+	+	+	+
8325	100±17·10	0	16·29±26·71*	115·45±0·48	30·62±26·06*	124·15±26·57	–	+	+	+
8345	100±13·10	0	62·74±12·17*	114·97±5·88	35·36±29·28*	85·72±18·50	+	+	+	+
8347	100±7·64	0	37·54±15·28*	19·93±11·29*	73·08±18·93	93·02±16·27	+	+	+	+
8348	100±4·90	0	32·56±4·65*	123·83±3·27	57·21±11·16*	113·08±24·76	+	+	+	+

*Significantly different from the control ($P<0·05$)

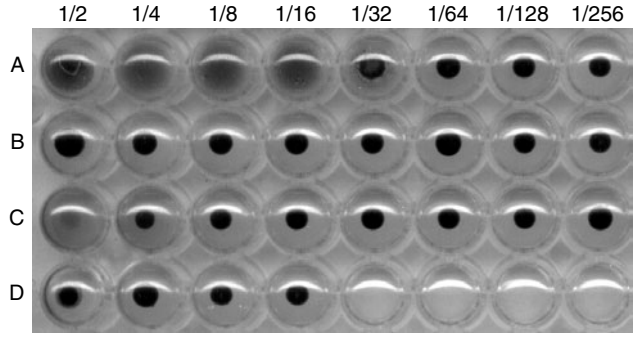

Fig. 4. Haemagglutination in presence of *Lb. kefir* CIDCA 8321 (A), *Lb. kefir* CIDCA 8321 treated with 5 M-LiCl (B), purified S-layer from *Lb. kefir* CIDCA 8321 (C) and control of red blood cells (D).

(Golowczyc et al. 2007). We previously reported that removal of S-layer proteins abolished the autoaggregation capability of *Lb. kefir* CIDCA 8321 (Garrote et al. 2004). In the present study the treatment of aggregating *Lb. kefir* strains with LiCl or SDS drastically diminished percentages of co-aggregation, suggesting an important role for S-layer proteins in bacteria-yeast interaction.

The reduction of co-aggregation percentage observed when *Lb. kefir* S-layer proteins were added to a mixture of *Lb. kefir* CIDCA 8321 and *Sacch. lipolytica* CIDCA 812 showed a competition between free protein and bacterial surface for the attachment to yeast cells. The increasing in aggregation percentage of yeast when purified *Lb. kefir* S-layer proteins were added to a suspension of *Sacch. lipolytica* CIDCA 812 provides additional support for the idea of a direct protein-yeast interaction. The failure of the performed proteolytic treatments to inhibit the co-aggregation could be attributed to a high resistance of bacterial S-layer against hydrolysis by proteases, as was previously observed for other S-layers (reviewed in Engelhardt & Peters, 1998).

Roos & Jonsson (2002) described the influence of pH on the affinity of *Lb. reuteri* surface proteins for carbohydrate structures in mucus and Kos et al. (2003) reported a pH dependence in auto-aggregation of *Lb. acidophilus*. In contrast, in the present work the lack of influence of pH on co-aggregation between *Lb. kefir* and *Sacch. lipolytica* suggests that ionic interactions are not involved.

Lectins are proteins or glycoproteins of non-immunological origin that bind to specific carbohydrates. Their first use as cytological tools was for differential binding of human erythrocytes in blood typing (Boyd et al. 1954) and several works about their role in different types of interactions (bacteria-bacteria, biofilm formation, bacteria-yeast, bacteria-host cell) have been reported (Roos & Jonsson, 2002; Tielker et al. 2005; Sun et al. 2007). The role of lectins in haemagglutination phenomena is well known (Mukai et al. 1998) and recently Uchida et al. (2006a) described a surface lectin activity in lactic acid bacteria with the ability to bind to ABO antigens expressed in intestinal mucosa. The ability of the co-aggregating *Lb. kefir* strains studied in the present work to agglutinate human erythrocytes, together with the loss of this ability in *Lb. kefir* after removal of S-layer proteins and the ability of purified S-layer protein to induce haemagglutination allowed us to hypothesize that S-layer proteins have a lectin-like activity. The fact that co-aggregation between lactobacilli-yeast was significantly decreased in the presence of different sugars and that yeast pretreated with sodium periodate (which oxidizes surface carbohydrates) became non co-aggregative strongly support our hypothesis. Significant decrease in co-aggregation of five out of six aggregative lactobacilli strains in presence of mannose could be attributed to the presence of mannans in the yeast surface.

Other authors also reported a lectin-like activity of surface proteins in probiotic strains of lactobacilli (Lakhtin et al. 2006) and co-aggregation mediated by bacterial surface peptides and yeast carbohydrates (Ocaña & Nader–Macías, 2002), but to our knowledge a lectin-like activity of S-layer proteins in *Lactobacillus* genus was only reported by Uchida et al. (2006b) for *Lb. brevis* strains with ability to bind to human A-antigen in intestinal mucosa.

The present work is the first report of a strain-specific lectin-like activity of S-layer proteins involved in the interaction between microorganisms. It contributes to the study of the relations among lactobacilli and yeast present in a complex microbial ecosystem as the kefir grain, in particular the co-aggregation of *Lb. kefir* and *Sacch. lypolitica*. Taking into account the complexity of this community, further studies are needed to demonstrate the specific role of this kind of interaction in the ecology of the kefir grains and the technological and probiotic properties of kefir-fermented products.

A.G. Abraham, G.L. Garrote and M. A. Serradell are members of the Carrera del Investigador Científico y Tecnológico of CONICET, G.L. De Antoni is a member of the Carrera del Investigador Científico y Tecnológico of CIC-PBA. M. Golowczyc and P. Mobili are fellows of CONICET. This work was supported by Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), CONICET, CIC-PBA and Universidad Nacional de La Plata (UNLP).

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