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S-layer proteins from *Lactobacillus* sp. inhibit bacterial infection by blockage of DC-SIGN cell receptor

Mariano Prado Acosta [a,*,1], Sandra M. Ruzal [a], Sandra M. Cordo [b,*,1]

[a] Laboratorio de Bacterias Gram Positivas, Departamento de Química Biológica-IQUIBICEN, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires-CONICET, Argentina

[b] Laboratorio de Virología, Departamento de Química Biológica-IQUIBICEN, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires-CONICET, Argentina

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ABSTRACT

Many species of *Lactobacillus* sp. possess Surface(s) layer proteins in their envelope. Among other important characteristics S-layer from *Lactobacillus acidophilus* binds to the cellular receptor DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin; CD209), which is involved in adhesion and infection of several families of bacteria. In this report we investigate the activity of new S-layer proteins from the Lactobacillus family (*Lactobacillus acidophilus, Lactobacillus brevis, Lactobacillus helveticus* and *Lactobacillus kefiri*) over the infection of representative microorganisms important to human health. After the treatment of DC-SIGN expressing cells with these proteins, we were able to diminish bacterial infection by up to 79% in both gram negative and mycobacterial models. We discovered that pre-treatment of the bacteria with S-layers from *Lactobacillus acidophilus* and *Lactobacillus brevis* reduced bacteria viability but also prevent infection by the pathogenic bacteria. We also proved the importance of the glycosylation of the S-layer from *Lactobacillus kefiri* in the binding to the receptor and thus inhibition of infection. This novel characteristic of the S-layers proteins may contribute to the already reported pathogen exclusion activity for these *Lactobacillus* probiotic strains; and might be also considered as a novel enzymatic antimicrobial agents to inhibit bacterial infection and entry to host cells.

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1. Introduction

Many species of the genus *Lactobacillus* possess Surface layer (S-layer) proteins. This macromolecular complex is an array of single (glyco)proteins non-covalently bound that constitute the outermost cell envelope of almost all Archaea and many Bacteria. S-layer from many species of the genus *Lactobacillus* has been studied showing a great structural variety among them with few characteristics in common. Particularly, *Lactobacillus* S-layer proteins differ from those of other bacteria in their smaller size and high predicted pI [1]. Although many studies focus on the biochemistry of the S-layers, the actual relationship between biological functions and structure remains poorly understood. Different articles reported functions that vary from protective coat, maintenance of cell shape and adhesion, to modulation of cytokines and immunogenic factors [2–5]. Other micro integral membrane protein, similar to S-layers, has been shown to adhere to mucin and antagonize the adhesion to epithelial cell of member of *Enterobacteriaceae* family [6,7].

Probiotics are live microorganisms, usually contained in food, traditionally regarded as safe for human consume. From many *Lactobacillus* species reported to be probiotics, several possess S-layer proteins, such as *Lactobacillus brevis* that possess a S-layer protein which exhibited an antiviral activity against Herpes virus-2 [8]. Other S-layer from *Lactobacillus plantarum* has been reported to protect intestinal epithelial cells injuries induced by enteropathogenic *Escherichia coli* [9]. Additionally, we have showed a protective, antiviral effect of the S-layer of *Lactobacillus acidophilus* against an important human pathogen, Junín virus, by blocking the entry of the virus to host cells via DC-SIGN receptor [10]. This novel activity increased the already probiotic status reported for *Lactobacillus acidophilus*.

DC-SIGN is a trans-membrane protein, C-type calcium-dependent lectin that can capture antigens for processing and presentation of both viral and bacterial nature. Virus including HIV-1, Ebola, Junín and Dengue [11–14]; and important bacteria such

* Corresponding authors at: Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, UBA, Ciudad Universitaria, Pabellón 2, Piso 4, 1428 Buenos Aires, Argentina.

E-mail addresses: m.pradoacosta@gmail.com (M. Prado Acosta), scordo@qb.fcen.uba.ar (S.M. Cordo).

[1] Both contributed equally to this work.

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as *Mycobacterium tuberculosis* have been previously shown to be recognized by this lectin [15–17].

DC-SIGN is highly expressed in dendritic cells [18] and it is reported to bind to lipopolysaccharide component from several Enterobacteriaceae such as *Escherichia coli, Shigella spp., Salmonella spp* [19–21] and *Klebsiella spp* [22]. The bacteria use DC-SIGN as an adhesion factor and are also involved in phagocytosis. Regarding *Mycobacterium tuberculosis*, components of the mycobacterial cell wall bind to DC-SIGN on dendritic cells and macrophages [23–25] and this interaction initiates antigen presentation and subsequent immune responses [26].

S-layer from *Lactobacillus acidophilus* was shown to bind to the receptor DC-SIGN [27] therefore in the present work we decided to extend our studies on *Lactobacillus* S-layers and their properties to block binding of important microbial pathogens to DC-SIGN lectin. From our previous report we chose *Lactobacillus acidophilus* S-layer but also added S-layers from *Lactobacillus brevis* and *Lactobacillus helveticus* that are important to food industry. Lastly S-layer from *Lactobacillus kefiri* was also included in this study which, besides being used in many fermented food, possess a glycosylated S-layer that may interact with the lectin. We purified and evaluated how these proteins interfere with the infection of four microorganisms of health importance: three enterobacteria and *Mycobacterium smegmatis*, a non-pathogenic model for *Mycobacterium* infection.

2. Materials and methods

2.1. Bacterial strains and cells culture conditions

3T3 cells (ATCC CCL 1658) and 3T3hDC-SIGN expressing cells (NIAID AIDS Research and Reference Reagent Program) were grown in Eagleís minimal essential medium (MEM) supplemented with 10% Fetal bovine serum (FBS). In all cases, cultures were grown at 37 °C in 5% of CO2. *Lactobacillus acidophilus* (*Lb. acidophilus)* ATCC4356; *Lactobacillus brevis* (*Lb. brevis*) ATCC 14869; *Lactobacillus helveticus* (*Lb. helveticus*) ATCC12046 and *Lactobacillus kefiri* (*Lb. kefiri*) JCM5818 were grown in MRS medium. *Escherichia coli* (*E. coli*) HB101, *Salmonella enterica serovar typhi* (*S. typhi)* and *Klebsiella pneumoniae* (*K. pneumoniae)* were grown in LB medium and *Mycobacterium smegmatis* mc^2 155 (*M. smegmatis*) in 7H9 medium with addition of albumin-dextrose-catalase (ADC) Growth Supplement (Sigma-Aldrich).

2.2. Isolation of S-layer proteins

The S-layer proteins were extracted from overnight *Lactobacillus* cultures grown in MRS medium at 37 °C, by using 6 M lithium chloride, extensively dialyzed against distilled water overnight at 4 °C and after centrifugation (10000*g*, 20 min) suspended in sterile PBS and store at −20 °C.

2.3. Cell-ELISA assays

3T3 and 3T3hDC-SIGN expressing cell cultures were seeded on 96 well microplate, grown to confluency, fixed with paraformaldehyde (PFA) 4% at room temperature (RT) for 15 min, washed, dried and frozen until used. Only wells with complete monolayers were used for the analysis. Cells were blocked with bovine serum albumin (BSA) 3% (1 h at RT), washed, incubated with PBS or mannan (Sigma-Aldrich) 100 μg/ml (1 h at RT) and washed again. Then, cell monolayers were incubated with antibody anti-S-layer of *Lb. acidophilus* (1 h at RT) to recognize bound S-layer to cells. Anti-mouse HRP antibody was used as secondary antibody and reading of the microplate was performed at 450 nm in an ELISA reader. In competition experiments cells were first incubated with different S-layers at a concentration of 50 μg/ml, then washed and incubated with S-layer from *Lb. acidophilus* and finally specific antibody and secondary antibody was used as mentioned above.

2.4. Cell-associated infectivity assays

Invasiveness of the indicated bacterial strains was determined using a gentamicin protection assay as described previously [28]. Briefly, 3T3 and 3T3hDC-SIGN cells were pre-treated with the indicated S-layers (400 μg/ml) and then infected at a multiplicity of infection (MOI) of 50 with infective strains pre-cultured in the appropriate broth. After 1 h, cell monolayers were washed and media containing 40 μg/ml of gentamicin was added for 90 min. Diluted cell lysates (0.5% Triton-X-100) were spread on LB agar plates to determine the number of cell-associated colony forming units (CFU) per well. Bacterial invasiveness was calculated as percentage of recovered cell-associated bacteria compared with different treatments and controls.

2.5. Bactericidal assays

Bacterial cells were grown until optical density (OD) = 1 and then washed twice with PBS. The bacterial suspension was treated with the indicated S-layer at 400 μg/ml and were incubated at 37° C for 1 h. After this pre-treatment both, bacteria viability was measured by direct CFU counting and cell-associated infectivity was quantified as described previously in 2.4.

2.6. Zymograms

Zymography was performed as previously described [29,30]. Polyacrylamide gels were cast with 0.01% sodium dodecyl sulfate (SDS) and protein mobility confirmed by coomassie blue stain. After the run of 10 μg/ml of each S-layer, hydrolase activity was detected by a clear zone.

2.7. De-glycosylation and glycoprotein staining

80 μg of purified S-layer were deglycosylated by PNGaseF treatment according to manufacturer's instructions (*NEW ENGLAND* Bio Labs). Briefly sample was denatured at 100 °C during 10 min and then treated with 5000 U of the enzyme for 3 h a 37 °C. The resulted sample was evaluated by SDS-polyacrylamide gels mobility. Similarly a control S-layer solution was mock treated and incubated at the same condition but in absence of the enzyme. For tunicamycin treatment *Lb. kefiri* strains were cultured in MRS broth with tunicamycin (20 μg/m1)(Sigma-Aldrich) at 30° for 24 h before S-layer extraction.

Glycosylation detection was performed with Pro-Q_Emerald 300 Glycoprotein Gel Stain Kit (Invitrogen) on SDS–polyacrylamide gels.

2.8. Statistical analysis

All the statistical analysis including inhibitory concentration (IC50) values were performed with the GraphPad Prism program. Values from at least three independent experiments were analysed by using *t*-test with a $p < 0.05$(*).

3. Results

3.1. Binding of the S-layer proteins to DC-SIGN

According to previous studies S-layer from *Lb. acidophilus* has the ability to recognize and bind the lectin human (h)DC-SIGN [27]. In the present work we developed a cell-ELISA assay

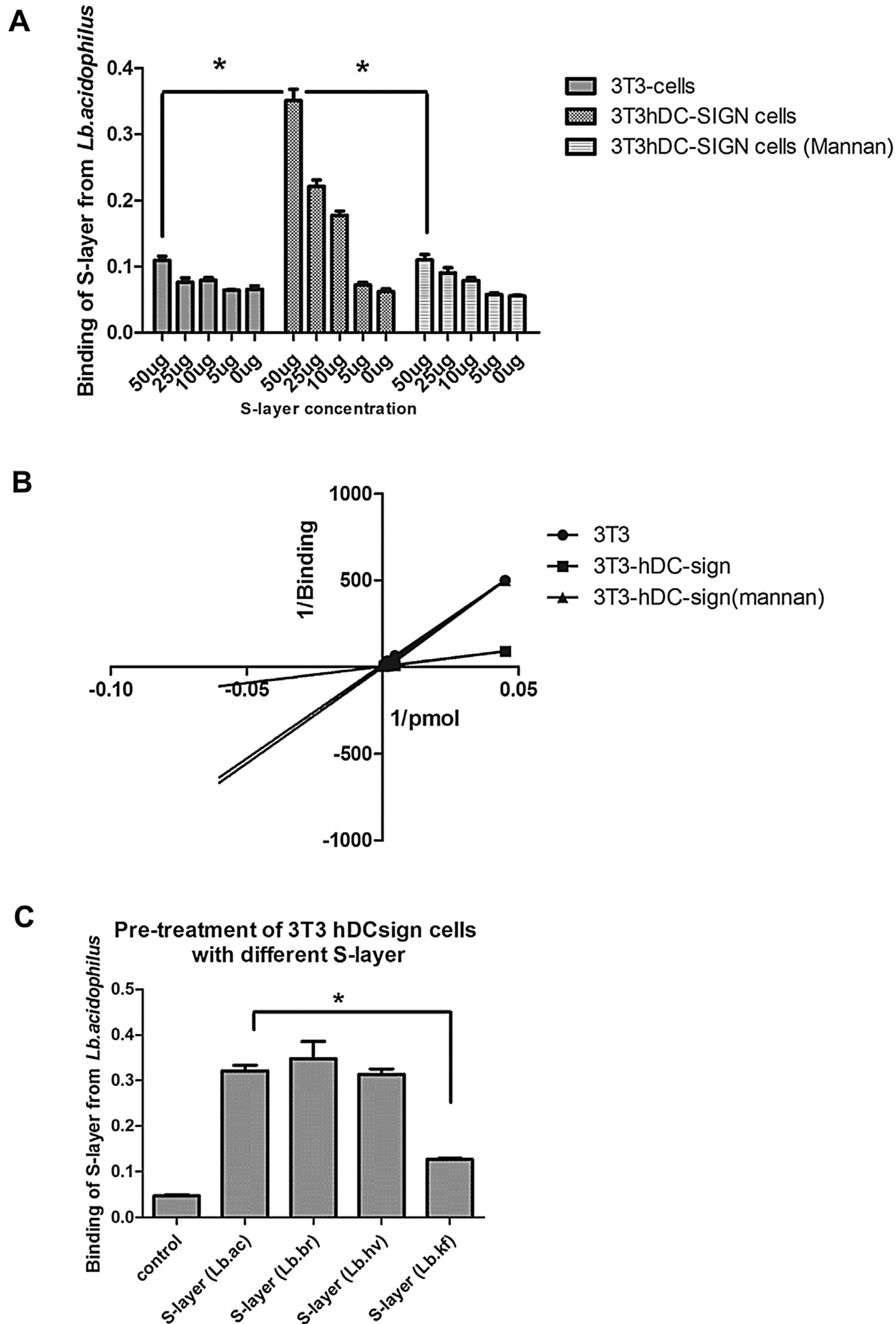

Fig. 1. (A) Binding of S-layer from *Lb. acidophilus* to cells. Fixed 3T3hDC-SIGN monolayers were incubated with increasing amounts of the S-layer. After several washes bound S-layer was determined by cell-ELISA assay. Non-expressing hDC-SIGN cells and mannan-blocked hDC-SIGN expressing cells were used as controls. (B) Double-reciprocal plot of the binding capacity of S-layer from *Lb.acidophilus* to hDC-SIGN. Data from Fig. 1A was transformed and plotted in order to obtain an apparent affinity constant (C) Competition between S-layer from *Lb. acidophilus* (Lb. ac) and S-layers from: *Lb. brevis* (Lb. br), *Lb. helveticus* (Lb. hv) and *Lb. kefiri* (Lb. kf). Fixed 3T3-hDC-SIGN monolayers were pre-incubated with each of the S-layers as depicted in the figure. Then all cultures were washed, incubated with S-layer from Lb. ac and its binding was revealed. Values from three independent experiments were analysed by *t*-test with a $p < 0.05$(*).

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to better determine recognition and binding of S-layers to this lectin. 3T3hDC-SIGN fixed cell monolayers were incubated with increasing amounts of S-layer. After incubation bound protein was detected using anti S-layer (*Lb. acidophilus*) antibody produced in our laboratory [31]. As shown in Fig. 1A, specific binding of S-layer from *Lb. acidophilus* to the 3T3 lectin-expressing cells was significantly different comparing with control: 3T3 non-lectin expressing cells. Moreover the binding to 3T3hDC-SIGN cells, measured by this method, showed to be S-layer dose-dependent. Importantly when hDC-SIGN was blocked by pre-incubation of the cells with mannan, specific S-layer binding was reduced to basal-control levels, as expected and previously reported [10].

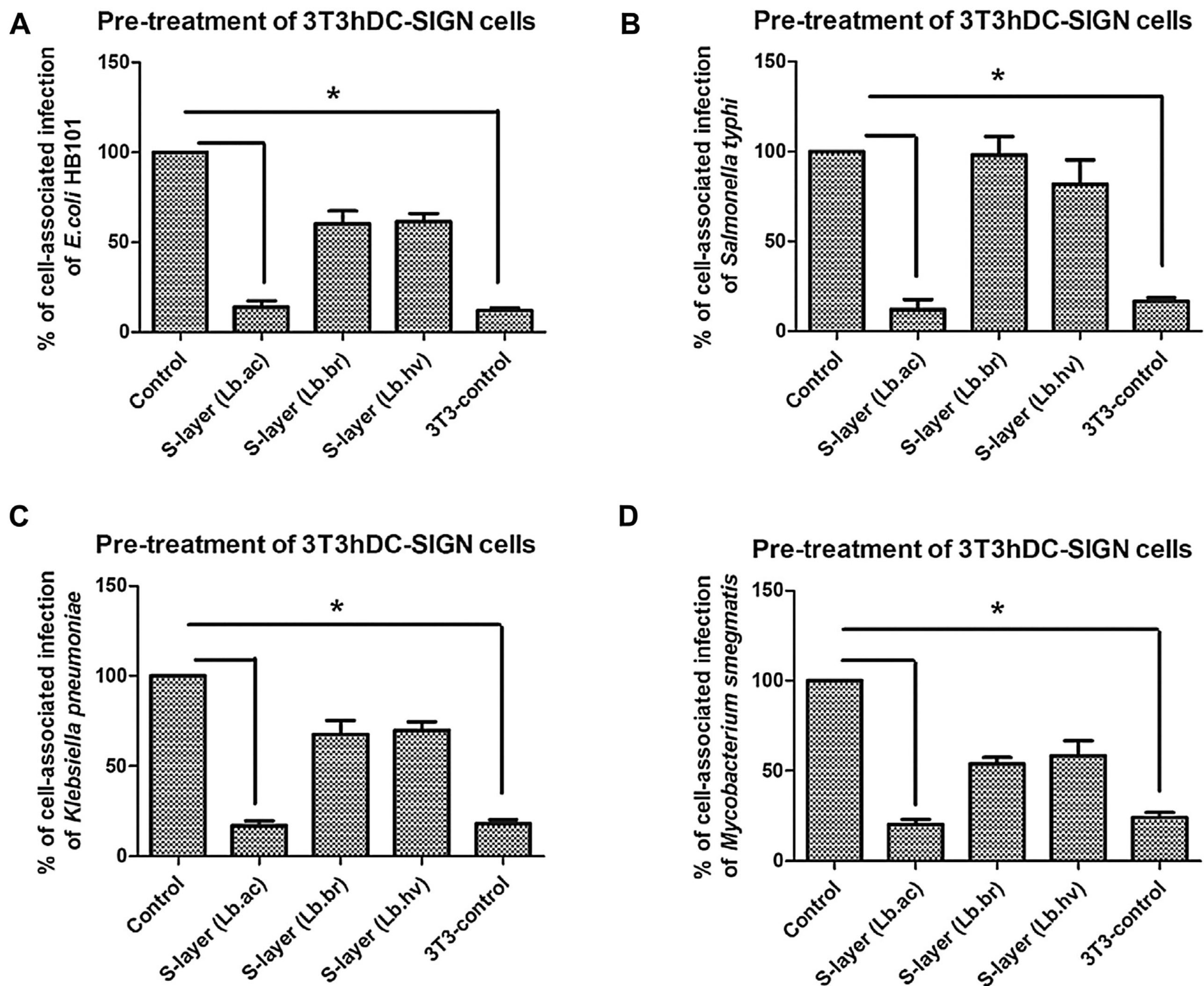

Fig. 2. Bacteria cell-associated infection in 3T3hDC-SIGN cultures pre-treated with S-layers. 3T3hDC-SIGN cells were pre-treated with different S-layer proteins and then infected with *E. coli* (A), *S. thypi* (B), *K. pneumoniae* (C) and *M. smegmatis* (D). After infection cell-associated infectivity was determined by CFU counting. Non S-layer treated cultures and 3T3 non-expressing hDC-SIGN cells were added as controls. Values from three independent experiments were analysed by *t*-test with a $p < 0.05$(*).
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In order to have a quantitative parameter to asses S-layers binding to hDC-SIGN receptor we used double-reciprocal plot transformation of data. Fig. 1B shows the transformed data of S-layer from *Lb. acidophilus*. Plotting of the binding capacity vs. protein concentration in a similar way to the drug-receptor interaction enables to transform the data in the double-reciprocal plot; that is valid in the analysis of dose-response assuming the classical theory in a drug-receptor interaction [32] Comparison of the three described conditions in Fig. 1B allows to estimate an apparent affinity constant (K_{ap}). K_{ap} values in a range of 0.5 μM were obtained for hDC-SIGN −S-layer interaction, this constant increased up to 11 μM when the receptor is blocked by mannan. From these data we concluded that mannan has a competitive inhibitory effect over S-layer for the DC-SIGN receptor.

The lack of antibodies against different S-layers is a common issue and, although we have previously developed an specific antibody that recognized S-layer from *Lb. acidophilus*, antibodies against the others S-layer are not commercially available. Then we make use of this unique tool to first determine whether S-layers isolated from *Lb. brevis*; *Lb. helveticus* and *Lb. kefiri* exhibited hDC-SIGN binding. We designed an in-plaque competition assay making use of the above described cell-ELISA assay. Fixed 3T3hDC-SIGNcells were first incubated with each of the competitive S-layer depicted in Fig. 1C. After 1 h monolayers were incubated with S-layer form *Lb. acidophilus* and after several washes bound S-layer was assessed by specific antibody against S-layer (*Lb. acidophilus*). The output values represented the capacity of each of the probed S-layers to specifically occupy and prevent S-layer (from *Lb. acidophilus*) binding to hDC-SIGN. As can be seen in Fig. 1C, S-layers from *Lb. brevis* and *Lb. helveticus* did not show any degree of competition while *Lb. kefiri* was the most effective protein in totally prevent S-layer from *Lb. acidophilus* from binding to hDC-SIGN.

3.2. Bacterial infection of 3T3hDC-SIGN cells treated with S-layers proteins

Ours results prompted us to evaluate the functional putative property of each of the S-layer proteins to interfere with important microorganisms known to attach to hDC-SIGN lectin. 3T3hDC-SIGN cultures were pre-treated with different S-layers, infected with *E coli, K. pneumoniae* or *S. typhi* and then cell-associated infectivity was quantified by CFU assays. Basal bacterial infection was determined in parallel infection of 3T3 parental cells. Fig. 2 shows that both, S-layer from *Lb. brevis* and *Lb. helveticus*, exhibited very

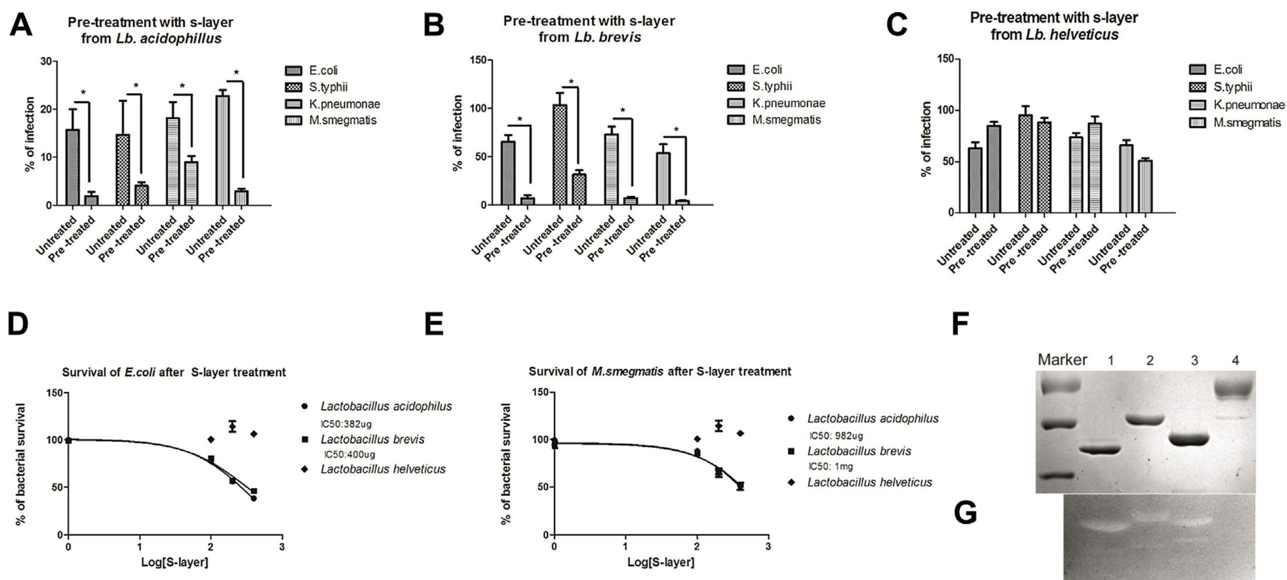

Fig. 3. Bacteria pre-treatment with S-layers. *E. coli* and *M. smegamtis* were pre-treated with S-layers from *Lb. acidophilus* (A), *Lb.brevis* (B) and *Lb. helveticus* (C) and then cell-associated infectivity was analysed by CFU counting. Cell-associated infectivity of control non-treated bacteria are depicted for each experiment. Values from three independent experiments were analysed by *t*-test with a $p < 0.05$(*). Viability of *E. Coli* (D) and *M. smegmatis* (E) after the treatment with different concentrations of S-layers.100% of survival corresponded to non-S-layer treatment condition. After pre-treatment of the bacteria with the indicated S-layers percentage of survival was quantified by direct CFU counting and IC50 was calculated. Zymogram (G) of the S-layer from *Lb. acidophilus* (line 1); *Lb. brevis* (line 2), *Lb. helveticus* (line 3) and *Lb. kefiri* (line 4). Coomassie blue of the corresponding zymogram (F).

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modest effect over infection of *E. coli* and *K. pneumonie;* while no apparent effect over *S. typhi* infection was seen. On the other hand pre-treatment of cells with S-layer from *Lb. acidophilus* showed a significant inhibition over all three microorganisms, reducing infection rates to basal levels. This 80% reduced infection rates were calculating comparing to control hDC-SIGN expressing cells. In all the experiments basal infection was determined over 3T3 parental cells and reached average values of 10–15%.

Besides the classical gram negative bacteria we also tested *Mycobacterium* since hDC-SIGN is the major *Mycobacterium tuberculosis* receptor on human dendritic cells [23]. We decided to use *M. smegmatis*, a non-pathogenic strain commonly used as an infection model of related *M. tuberculosis* infection. Fig. 2D shows that S-layer from *Lb. acidophilus* is the most effective inhibitor of *M. smegmatis* infection. Again the presence of S-layer from *Lb. acidophilus* exerted a significantly effect over infection, reducing levels to basal infection values.

3.3. Bactericidal activity of S-layers

To further define the action of these proteins we investigated the ability of all the four studied S-layers to alter bacterial viability. This time microorganisms were pre-treated with S-layers as explained in section 2.5. 3T3hDC-SIGN cells were infected and finally cell-associated infectivity was quantified as in Fig. 2. As depicted in Fig. 3 significantly lower levels of infection were obtained from cells infected with the pre-treated bacteria. This important change in the infection rate was observed when the bacteria were pre-treated with S-layer from *Lb. acidophilus* (Fig. 3A) and *Lb. brevis* (Fig. 3B), but no with *Lb. helveticus* (Fig. 3C). Previously we have showed that S-layer from *Lb. acidophilus* possess an hydrolytic activity against gram negative bacteria [31] and we presumed that S-layer from *Lb. brevis* may share this characteristic. To assess this we measured the viability of *E. coli* and *M. smegmatis* after S-layer pre-treatment at different concentrations by traditional plaque assay. Fig. 3D, E showed that the survival of the probed bacteria diminished after treatment with S-layers from *Lb. acidophilus* and *Lb. brevis*; but no with S-layer from *Lb. helveticus* and that this effect depends on the concentration of S-layer added. Inhibitory concentrations (IC50) was calculated for each protein showing similar values for the same group of bacteria as depicted in Fig. 3D, E.

To further analyse if the diminish in the bacterial survival was due to an hydrolytic activity, a zymogram with bacterial cells as substrate was performed. As previously reported S-layer from *Lb. acidophilus* exhibited a pronounced hydrolytic activity over substrate. Similarly we reported for the first time that S-layer from *Lb. brevis* also showed an hydrolytic capacity.

Even though an hydrolytic band was revealed in the zymogram, no apparent activity was found for the S-layer from *Lb. helveticus* in CFU counting, suggesting that elements in *in-vivo* assays may be blocking the activity. No activity was found in the S-layer from *Lb. kefiri* (Fig. 3G).

3.4. S–layer of Lactobacillus kefiri and glycosylation

From all four S-layer studied only S-layer from *Lb. kefiri* is known to be glycosylated [33] Results in Fig. 1B showed that S-layer from *Lb. kefiri* was the most effective protein in preventing S-layer from *Lb. acidophilus* binding to hDC-SIGN and most probably due to interaction with the same binding site on hDC-SIGN molecule. To further investigate the biological role of this result, infection assays were performed as in Fig. 2. For all tested bacteria infectivity values reached only 20% comparing with control non pre-treated hDC-SIGN expressing cultures (Fig. 4A). On the other hand pre-treatment of bacterial suspensions showed no effect over viability indicating non-bactericidal activity (Fig. 4B). We then hypothesized that the sugar decoration from *Lb. kefiri* S-layer might be involved in lectin recognition and bacterial infection inhibition. To test this we produced S-layers in two different conditions known to affect glycosylation. First, bacteria *Lb. kefiri* was grown in the presence of tunicamycin and then the S-layer further purified. Second, S-layer from *Lb. kefiri* normally produced and purified was enzymatic treated by PNGaseF. The effect of both treatments over S-layer from *Lb. kefiri* glycosylation was first visualized by a glycosyla-

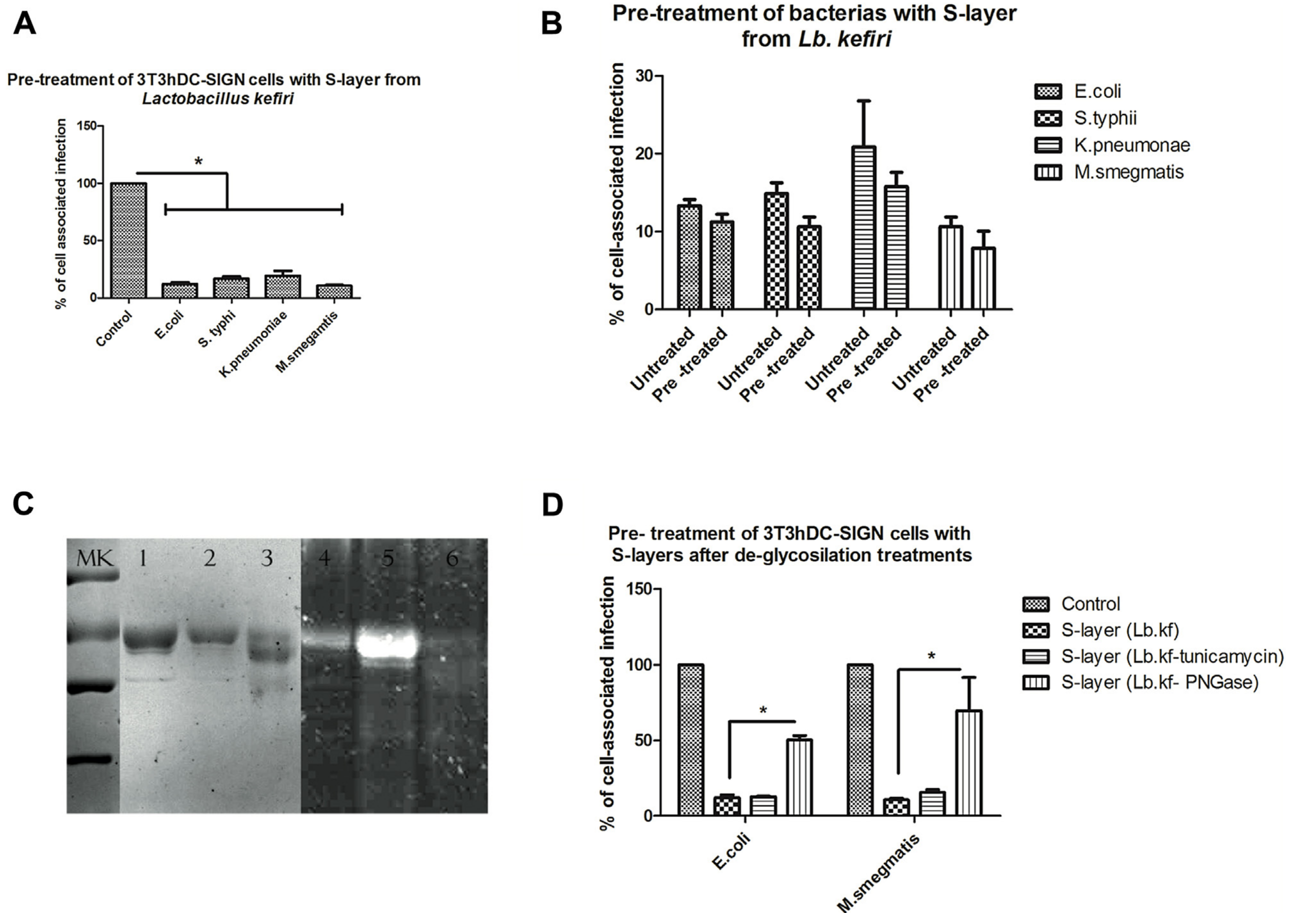

Fig. 4. Bacteria cell-associated infectivity in 3T3hDC-SIGN cultures pre-treated with S-layer from *Lb. kefiri* (A). Different bacteria were used to infect cell cultures pre-treated with S-layer from *Lb. kefiri* and then cell-associated infectivity was calculated. Non-treated culture cells were infected with the depicted bacteria and cell-associated infectivity set as 100% as control. Values from three independent experiments were analysed by *t*-test with a $p < 0.05$(*). Bacterial pre-treatment with S-layers from *Lb. kefiri* (B). Bacteria were pre-treated and then cell-associated infectivity was analysed. Cell-associated infectivity of control non-treated bacteria is depicted for each experiment. De-glycosylation of S-layer from *Lb. kefiri* (C). Control (line 1 and 4), tunicamycin (line 2 and 5), PNGaseF enzymatic treatment (line 3 and 6). Coomassie blue stain (left panel) shows the shift on glycosylation pattern and Pro-Q emerald kit (right panel) reveals glycosylated proteins. Bacteria cell-associated infectivity in 3T3hDC-SIGN cultures pre-treated with de-glycosylated S-layer from *Lb. kefiri* (D). Bacteria were used to infect cell cultures and cell-associated infectivity was calculated as in (A).
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tion kit according to manufacturer's protocols (Fig. 4C). Although exact composition of S-layer glycosylation is not known we demonstrated that enzymatic treatment did affect protein mobility while tunicamycin apparently did not (Fig. 4C, right panel). Next, both sources of S-layer from *Lb. kefiri* were used to perform infection assays. As shown in Fig. 4E *E. coli* infection was restored by 50% in 3T3hDC-SIGN cells pre-treated with S-layer from *Lb. kefiri* that was previously incubated with the enzyme PNGaseF. Similar results were observed over *M. smegmatis* infection. On the other hand tunicamycin treatment had no effect on S-layer activity (Fig. 4D).

4. Discussion

Infectious diseases now need the development of totally new classes of antibacterial agents, ones that cannot be thwarted by the same gene products that render bacteria resistant to antibiotics. Within this enzybiotics or peptide antibiotics that act as enzymatic antimicrobial agents, S-layers are promising candidates. Previously we have found that S-layer protein of *Lb. acidophilus* possess properties that enable us to postulate it as antiviral and antibacterial candidate [10,31]. In the present work we showed that distinct S-layers have different ability to reduce the infection of relevant enterobacteria and also mycobacteria. S-layer from *Lb. acidophilus* exerted this effect throughout a possible combined action that involved blockage of hDC-SIGN (Fig. 2) and direct bactericidal activity (Fig. 3D and E). On the other hand S-layer from *Lb. brevis* exhibited poor effect over infection of hDC-SIGN expressing cells. The fact that this S-layer showed to reduced bacterial viability revealed that the moderate effect over infection, was likely due it bactericidal activity rather than to DC-SIGN recognition. Binding experiments shown in Fig. 1 also supports this observation.

The S-layer structure is predicted to be fold in two well defined modules, one responsible for anchoring to the cell wall and including a catalytic module that hydrolyses the peptidoglycan, in the C-terminal. The N-terminus is assumed to be responsible for the interaction of monomers to form an external layer as well as the direct interaction with the environment [34–36].

When performing phylogenetic analysis for this study the four S-layer proteins used correlated with their evolutionary origin being *Lb. helveticus* (Accession No CAB46984.1) and *Lb. acidophilus* (Accession No CAA61560.1) more closely related and belonging to the *Lb. acidophilus* homology group. *Lb. brevis* (Accession No CAA78618) and *Lb. kefiri* (predicted from WGS, strain JCM5818.contig00016) showed high similarity in their amino terminal domains. Difference between both pairs of S-layer proteins at their primary amino acid sequence could explain the different behaviour observed regarding their ability to interfere with pathogen infection. On the other hand when analysing the bacterial infection of 3T3hDC-SIGN cells

treated with S-layers of *Lb. helveticus* and *Lb. acidophilus*, significant differences were also observed between them. However, the predicted S-layer protein of *Lb. helveticus* has 74% identity and 83% similarity with that of *Lb. acidophilus*. Differences in primary sequence in the N-terminus between S-layer proteins have been shown to result in cells that have weak immunomodulatory activity [33,34,38]. *Lb. acidophilus* CP23 with weak immunomodulatory activity lacks anchoring structure for surface layer protein [39] and this is probably the case in *Lb. helveticus* S-layer protein.

In the case of S-layer from *Lb. kefiri* compared to that of *Lb. brevis* the results obtained could be in part a consequence of the presence and absence of glycosylation respectively. While *Lb. brevis* is not glycosylated, in *Lb. kefiri* protein we observed a glycosylation signature motif SASSAS at the N-terminus. Here, we first showed that *Lb. kefiri* S-layer was surprisingly effective on preventing *Lb. acidophilus* bound to hDC-SIGN (Fig. 1). Afterwards, our studies showed that this S-layer has strong activity against infection of hDC-SIGN expressing cells with no appreciable bactericidal properties (Fig. 4A and B). On the other hand de-glycosylation by PNGasaF remarkable reduced its activity (Fig. 4D). All together this results suggest that S-layer from *Lb. kefiri* binds to hDC-SIGN and as a result exhibits an effective ability to diminish bacterial infection, by specifically blocking the usage of this receptor. Other authors have shown that diverse *Lb. kefiri* strains stimulate the production of different ratios of pro/anti-inflammatory cytokines *in vitro* [37], possibly but not only due to the capacity to engage DC-SIGN molecule on the surface of immune cells. It has also been shown that other S-layer like proteins from *Lactobacillus plantarum* bind to DC-SIGN and induce the maturation and production of anti-inflammatory cytokines of dendritic cells [6]. This, together with our present results on antimicrobial S-layer activity makes *Lb. kefiri* a good candidate to be used in gut inflammatory disorders.

The exact mechanisms by which the S-layer reduces infectivity of DC-SIGN interacting pathogens are yet not known. From our results however we can postulate various probable ways of action: the first way would involve bacterial direct inactivation through the hydrolytic cell wall activity that requires a functional anchoring structure for surface layer protein to the target bacteria. This is visualized by the different antibacterial effect found between closely related *Lb. acidophilus* and *Lb. helveticus*. Also *Lb. brevis* S-layer has the capacity to hydrolyse bacteria but to a lesser extent than that of *Lb. acidophilus*. Carbohydrate recognition domain (CRD) that mediates the recognition of carbohydrate structures found in multiple pathogens (e.g., HIV, Dengue virus, Lassa virus, Ebola virus, *M. tuberculosis, C. albicans, S. mansoni*, and *H. pylori*, among others) were predicted from the amino acid sequence in the C-terminal portion of the S-layer from *Lb. acidophilus* [7], and also observed in *Lb. helveticus* and the N-terminal of *Lb. brevis* [40]; implying that this domains could be actually active in recognizing carbohydrates pathogens structures. This second way to reduce infectivity would result by directly competing with the lectin. Since DC-SIGN mediated pathogen interaction involve the recognition of the glycans expressed on various pathogens, the glycans found in glycosylated S-layer protein like the one from *Lb. kefiri* might compete with the pathogen for the CRD on the DC-SIGN lectin.

We have not found evidence of glycosylation in the S-layers from *Lb. acidophilus* [10] neither from *Lb. helveticus* or *Lb. brevis* using the ProQ Emerald kit (data not shown), although some authors have reported them to be glycosylated by indirect detection techniques using plant lectins (i.e. Concanavalin A) interaction. Interaction between DC-SIGN and S-layer proteins, as that described with plant lectins [27] independent of glycosil motifs, is then a feature found for S-layer protein. If this is the case then this could be a third postulated mechanism to reduce pathogen infectivity.

Overall, despite similar carbohydrate binding sequence motifs in *Lactobacillus* S-layer proteins from the different species they show an enormous and differential biological potential to interact with both pathogens and host cells resulting in diminished microorganisms invasion. This and future studies would certainly contribute to add, to the well-known probiotic characteristics observed for the different strains, novel and still uncovered S-layer functions.

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