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Characterization of liposomes coated with S-layer proteins from lactobacilli

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Abstract

The stability of liposomes coated with S-layer proteins from *Lactobacillus brevis* and *Lactobacillus kefir* was analyzed as a previous stage to the development of a vaccine vehicle for oral administration. The interactions of the different S-layer proteins with positively charged liposomes prepared with soybean lecithin or dipalmitoylphosphatidylcholine were studied by means of the variation of the Z potential at different protein–lipid ratios, showing that both proteins were able to attach in a greater extent to the surface of soybean lecithin liposomes. The capacity of these particles to retain carboxyfluorescein or calcein by exposure to bile salts, pancreatic extract, pH change and after a thermal shock showed that both S-layer proteins increased the stability of the liposomes in the same magnitude. The non-glycosylated protein from *L. brevis* protects more efficiently the liposomes at pH 7 than those from *L. kefir* even without treatment with glutaraldehyde.

Keywords: S-layer protein; Liposome; Lactobacilli; pH stress; Thermal shock; Bile and pancreatic bile effect; CF and calcein release

1. Introduction

One of the commonly observed outer surface components of cell envelopes of prokaryotic organisms, archaea and bacteria, are crystalline arrays of proteinaceous subunits, known as surface layers (S-layers) [1–3]. S-layers are composed of single protein or glycoprotein species and represent the simplest biological membrane developed during evolution. Isolated S-layer subunits of numerous organisms are able to assemble *in vitro*, either in suspension, at liquid surface interfaces, on lipid films including liposomes and on solid supports [4].

Artificial lipid vesicles such as liposomes are widely used as delivery systems for enhancing the delivery into cells and tissues of different biologically active molecules encapsulated in the internal aqueous lumen or dissolved in the lipid bilayer itself [5,6]. Currently, liposomes are used as drug delivery vehicles to transport therapeutic agents to disease sites *in vivo*. These agents include low molecular weight drugs used in cancer chemotherapy and genetic drugs including plasmids encoding therapeutic genes [7].

Generally, liposomes release their contents when interact with target cells either by adsorption, endocytosis, lipid exchange, or fusion. In all these processes the interfacial properties of the liposomes play a fundamental role [6]. Due to the intrinsic adjuvant ability of S-layers [8] as well as their capability to surface-display proteins and epitopes [9,10] S-layer-coated liposomes are excellent candidates to be used as antigen carriers.

One of the problems faced in the use of liposomes as drug delivery system orally administered is that in their route they have to surmount the effect of different biological media. Among the changes of pH, exposure to bile salts, and pancreatic extracts can be named. In addition, the media may affect the response of the bilayer permeability to thermal shocks.

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Liposome stability towards different stress factors has been significantly enhanced when they were coated with S-layer proteins from *Geobacillus stearothermophilus* [11,12]. However, the possibility to prepare lipid particles using S-layers from microorganisms with beneficial effects for human health has not been reported yet.

The S-layer seems to be a typical surface structure in several *Lactobacillus* species, such as *L. acidophilus*, *L. helveticus*, *L. casei, L. brevis*, *L. buchneri*, *L. fermentum*, *L. bulgaricus* [12], *L. plantarum* [13], *L. kefir* [14] and *L. gallinarum* [15].

On SDS-gels, isolated S-layer proteins from *L. brevis* JCM 1059 exhibited an apparent molecular weight of 49.5 kDa [12], and isolated S-layer protein from *L. kefir* JCM 5818, exhibited a molecular weight of 69.0 kDa [14]. It is important to notice that, as demonstrated by periodic acid-Schiff staining, the S-layer protein from *L. kefir* is glycosylated (unpublished data).

This structural difference might be important from stability and surface properties.

The aim of this work is the characterization of the binding of S-layer protein from *L. brevis* and *L. kefir* on lipidic surfaces and the analysis of the stability in relation to the different composition of both S-layers.

For this study liposomes composed by dipalmitoylphosphatidylcholine (DPPC) or soybean lecithin (SL), with the addition of similar ratios of cholesterol and stearylamine, were used.

The stability after thermal shock, chasing by pancreatic extract and bile salts, and at different pHs mimicking the gastrointestinal track were tested by the release of vesicle content.

2. Materials and methods

2.1. Bacterial strains, growth conditions and isolation of the S-layer protein

Lactobacillus kefir JCM 5818 and *Lactobacillus brevis* JCM 1059 were grown to mid-log phase in 250 ml of MRS broth (Biokar Diagnostics, Beauvais, France) at 37 °C, harvested by centrifugation (5,000×*g*, 15 min, 4 °C), and washed twice in physiologic solution. The S-layer protein was extracted with guanidine hydrochloride solution (GHCl 5 M in 50 mM Tris–HCl buffer, pH 7.2) at 20 °C for 2 h. GHCl-extracted S-layer proteins were dialyzed against distilled water at 22 °C for 2 h. To assure the absence of large S-layer aggregates a centrifugation at 16,000×*g* for 20 min at 4 °C by a modification of Jahn-Schmid et al, 1996 protocol [8] was carried out. The solution obtained did not show turbidity and was employed to titrate the liposomes. The S-layer protein content of the clear supernatant was evaluated by SDS-PAGE 12.5% and its concentration was determined by the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, California, USA).

2.2. Preparation of liposomes with and without carboxyfluorescein (CF)/calcein

Positively charged liposomes were prepared from a mixture composed of 625 nmol of soybean lecithin (SL), 312 nmol cholesterol and 62.5 nmol stearylamine (Sigma, St. Louis, MO, USA). Another batch of liposomes was prepared replacing soybean lecithin by dipalmitoylphosphatidylcholine (DPPC) in the same ratio.

Lipids were dissolved in 2 ml chloroform and evaporated under nitrogen flow. The dry lipid film was rehydrated by addition of 1 ml PBS pH 7.0 and loosened from the glass wall during agitation above the transition temperature (45 °C) for 1 h. For the stability studies, carboxifluorescein-loaded SL liposomes were prepared by rehydrating the lipid film with PBS pH 7.0 supplemented with 11 mg/ml of 5 (6)-carboxyfluorescein (CF) (Molecular Probes, Eugene, OR, USA). At this concentration CF is self-quenched. Non-entrapped CF was removed from the external media after two centrifugations for 5 min at 5600×*g* and washed with PBS pH 7.0. For preparing calcein-loaded liposomes, the lipid film was rehydrated in a 65 mM calcein solution in 10 mM HEPES pH 7. Non-entrapped calcein was removed by the same procedure followed for CF.

S-layer coated liposomes containing CF/Calcein were prepared by incubation of 1000 nmol of total lipids with different concentrations of monomeric and/or oligomeric S-layer proteins. Adsorption of the S-layer proteins was carried out for 3 h at 22 °C under agitation.

To evaluate the effects of the S-layer protein on liposomes stability, the samples with different amount of proteins were incubated at 37 °C overnight. The percentage of enclosed CF was measured by the increase in the fluorescence after the addition of Triton X-100 exciting at 492 nm and monitoring the fluorescence at 518 nm.

With the aim to determine the minimal incubation time, an equivalent lipid mixture was incubated with S-layer protein of *L. kefir* or *L. brevis* to achieve a final concentration of 45 μg/ml and 200 μg/ml, respectively.

2.3. Electrophoretic mobility

Electrophoretic mobility of liposomes was determined in a Z-meter 3.0 (Zeta Meter Inc, Staunton, VA, USA) by applying a continuous electric field of 50 V to a liposome suspension in buffer. The movement of the particle in the electrical field was followed by microscopic visualization in a reticulated objective. Values of the electrophoretic mobility (μ) were automatically given by the instrument. The zeta potential in volts (ξ) was calculated by the Smoluchowski equation:

$$\xi = 4\pi\frac{\eta\,\mu}{D}$$

where η is the viscosity of the suspension at 20 °C, D is the dielectric constant of the solution at 20 °C and μ is the electrophoretic mobility of particles (micrometer/s per volt/cm).

2.4. Electron microscopy

Negative staining with unbuffered uranyl acetate (2% in distilled water) was performed in samples of liposomes coated with 45 μg/ml of S-layer protein from *L. kefir*. Samples were examined in a JEOL 1200 EX II transmission electron microscope (TEM) at 100 kV.

2.5. CF release from S-layer-coated liposomes after incubation at different temperatures

The fluorescence of CF released from different liposomes in the course of temperature shifts was monitored after an incubation of 60 and 120 min, in PBS buffer (NaCl 0.1 M; PO_4HNa_2 6 mM; PO_4H_2K 1 mM; KCl 2.5 mM) pH 7.0, at 37 °C and 50 °C, using a Luminescence Spectrometer LS 55 (Perkin-Elmer Corp./Applied Biosystems, California, USA) with an excitation and emission wavelength of 492 nm and 518 nm, respectively. Experiments were performed with control liposomes and with S-layer-coated liposomes. Finally, the remaining CF entrapped in the liposomes was released by addition of Triton X-100 solution (20% v/v in distilled water) to achieve a final concentration of 1%. The percentage of enclosed CF before incubation was determined by the following equation:

$$100 - \frac{(F - F_I) \times 100}{(F_T - F_I)}$$

Where F is the fluorescence of the sample after each incubation time, F_I is the fluorescence remnant of the sample immediately after the washing of the liposomes and before the incubation. F_T is the fluorescence of the sample

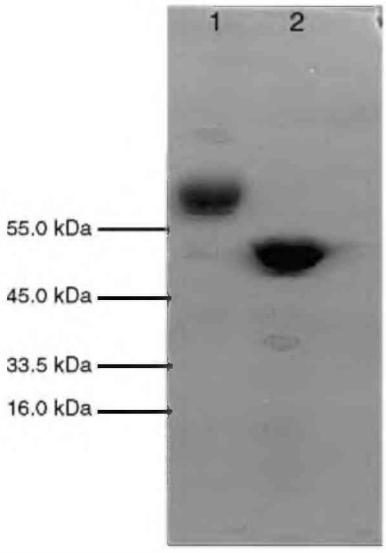

Fig. 1. SDS-PAGE patterns of *L. kefir* (lane 1) and *L. brevis* (lane 2) extracts after dialysis.

after the addition of Triton X-100, representing the 100% of encapsulated CF.

2.6. Glutaraldehyde treatment of S-layer coated liposomes

After its adsorption on liposomes, the S-layer lattice was crosslinked with glutaraldehyde under different conditions (0.5, 1.5 and 2.5% glutaraldehyde in 100 mM PBS pH 7.2 at 22 °C for 20 min). The reaction was stopped by the addition of Tris (final concentration of 0.5 M in the reaction mixture). After centrifugation of glutaraldehyde-treated liposomes at 16,000×*g* at 4 °C for 10 min, the pellet was washed with 100 mM phosphate buffer, pH 7.2. Cross-linking of the S-layer lattice was examined by extracting S-layer-coated liposomes with sodium dodecyl sulfate (SDS) (5% SDS in distilled water 5 min at 100 °C) and by applying the SDS-extract to SDS-PAGE.

2.7. Calcein release from S-layer coated liposomes by incubation at different pH buffers

Release of the fluorescent dye calcein was monitored with a Luminescence Spectrometer LS 55 (Perkin-Elmer Corp). Experiments were performed by incubation of S-layer-coated liposomes in the following buffers: pH 2.50, 10 mM formic acid, pH 4.0, 10 mM acetate, and pH 7.0, 10 mM HEPES/NaOH. All buffers contained 150 mM NaCl and 0.1 mM EDTA, and complete dye release was obtained by the lysis of the liposomes with Triton X-100 (1% final concentration). The percentage of the enclosed calcein before incubation was determined by the same equation as in the previous assay. Measurements were carried out in a thermostated cuvette (1 ml), under constant stirring. Samples were excited at 490 nm and the emission at 520 nm was collected at 90°.

2.8. CF release from S-layer-coated liposomes by incubation in bile salts and pancreatic extract

The fluorescence of CF released from liposome preparations after an incubation of 60 and 120 min in a bile salts solution (Britania, Buenos Aires, Argentina) (10 mM sodium collate, in PBS pH 6.2), and 2.8% pancreatic extract in 10 mM Tris–HCl, 145 mM NaCl pH 7.4 (ICN Biomedicals Inc. Ohio, USA), was measured in the same conditions of the previous assays.

3. Results

SDS-PAGE analysis of GHCl-extracted S-layer protein after dialysis for both lactobacilli strains is shown in Fig. 1. Single protein bands with an apparent molecular mass of 49.5 and 69 kDa were obtained from *L. brevis* and *L. kefir* respectively.

In order to quantify the adsorption of the different types of S-layer to different liposomes zeta potentials of the dispersed particles were carried out. When soybean liposomes were incubated with increasing amounts of the soluble S-layer protein, a clear decrease in the Z potential of the liposomes from the initial value of 78.6±7.1 mV was observed (Fig. 2). This decrease suggests an interaction of the S-layer proteins with the lipid surface causing a change in the sign of the charges exposed to the aqueous phase. The minimal Z potential values achieved with the different S-layer proteins assayed were negative in comparison to that of uncoated liposomes. The final amount of each protein necessary to achieve the same minimal value was different. SL liposomes needed 200 μg/ml of S-layer protein from *L. brevis* (Fig. 2A) to obtain the Z potential value of −17.2±2.2 mV. In contrast, for the same type of liposomes, 45 μg/ml of S-layer protein from *L. kefir* (Fig. 2B) were necessary to decrease the potential to −16.5±5.4 mV. A different response was found with DPPC liposomes. In this case, liposomes coated with S layer still show a positive potential value for the highest

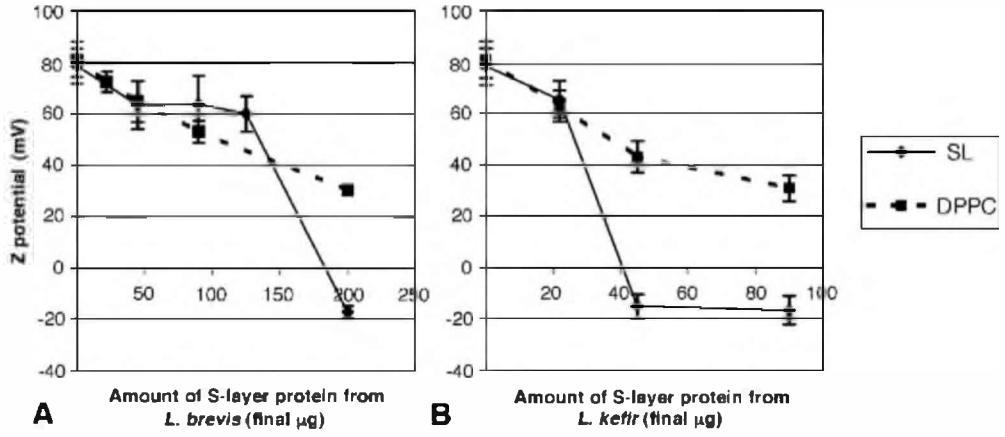

Fig. 2. Zeta potential values of multilamellar liposomes incubated with different amounts of S-layer proteins from *L. brevis* (A) and *L. kefir* (B). Each point represents the averages of twenty independent measurements in two different batches. Error bars indicate standard deviations of the means. All assays were carried out at 20 °C.

protein amount tested. The initial Z potential value was 81.1± 7.0 mV, at 25 °C, which is higher than that for liposomes prepared with SL. When DPPC liposomes were incubated with S-layer protein from *L. kefir*, the Z potential value decreased to 31.0±4.9 mV, and to 30.31±1.9 mV with S-layer protein from *L. brevis*.

The evaluation of the effect of incubation time over the vesicles showed that S-layer/ liposome interactions are time dependent. The minimal Z potential values were reached after an incubation of 150 min for both S-layer proteins. Longer incubation times did not show variation of Z potential values (Fig. 3).

When the influence of the amount of S-layer proteins added was evaluated, the highest CF retention capacity was obtained when SL liposomes were incubated with 45 μg of S-layer protein from *L. kefir*, and 200 μg of S-layer protein from *L. brevis* (Fig. 4). This is coincident with the minimal values achieved in the zeta potential (Fig. 2).

As a control of the interaction of S-layers with liposomes, electron micrographies for liposomes with and without S-layer proteins from *L. kefir* were carried out to verify the adsorption of S-layer proteins on liposome surface (Fig. 5). Although no details of the structure can be observed, the electron micrograph of negatively stained liposomes with S-layer protein corresponding to the zeta potential values of −16.5 mV (Fig. 2) shows a higher electronic density, in comparison to control lecithin liposomes.

3.1. In these conditions the effect of temperature on the encapsulation of CF was evaluated for both types of liposomes

Encapsulation of the hydrophilic fluorofore CF was performed by applying the dehydration–rehydration method [16]. The total amount of enclosed CF was determined by the fluorescence increase after the addition of Triton X-100 solution.

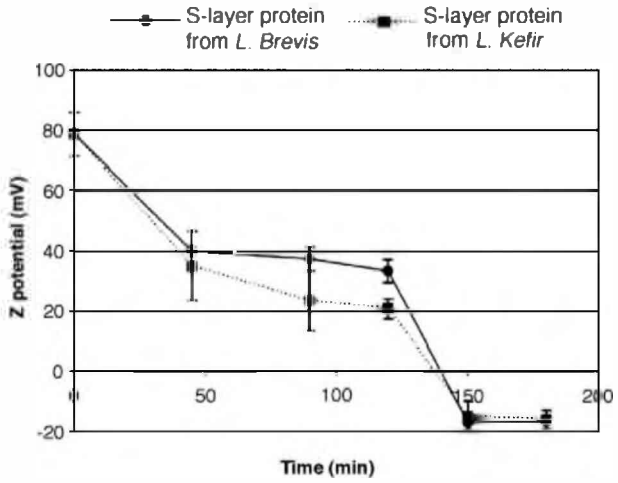

Fig. 3. Zeta potential values obtained after incubation of multilamellar liposomes with S-layer proteins from *L. kefir* y *L. brevis* at different times. Each point represents the averages of twenty independent measurements in two different batches. Error bars indicate standard deviations of the means. All assays were carried out at 20 °C.

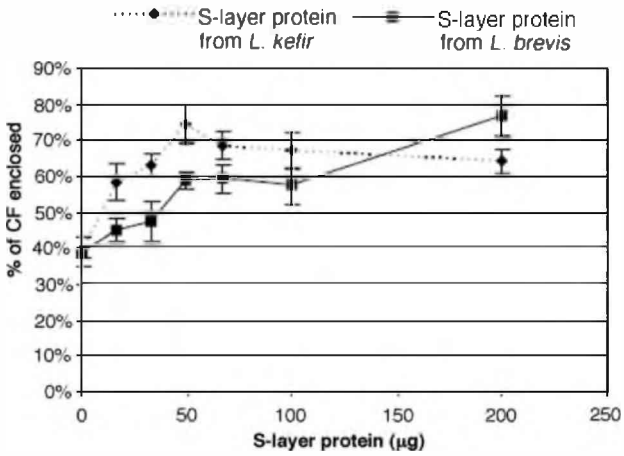

Fig. 4. Percentage of enclosed CF in soybean liposomes prepared with different amounts of S-layer proteins from *L. kefir* or *L. brevis* after incubation at 37 °C. The 100% of encapsulation was taken as that obtained by the addition of 10% Triton. Data represent the averages of three independent measurements from two batches of liposomes. Error bars indicate standard deviations of the means.

The entrapped CF is self-quenched at this high concentration. Thus, it does not contribute to the fluorescence of the samples [17]. The leakage of CF to the external media produces an increase in fluorescence due to the dequenching by dilution of the dye. As shown in Fig. 6, the percentages of enclosed CF after 60 and 120 min of incubation at 37 °C were 91.0 and 80.5%, respectively, for liposomes coated with *L. kefir* S-layer protein, and 91.5 and 77.3%, respectively, for liposomes coated with *L. brevis* S-layer protein. These percentages were significantly lower in the control liposomes (65.0 and 60.0% for each incubation time). At 50 °C, the percentages of enclosed CF after the incubation were 43.5 and 32.2% for each time, in liposomes coated with *L. kefir* S-layer protein. These values were 42.0 and 30.0%, respectively, in liposomes coated with *L. brevis* S-layer protein. The values for control liposomes at 50 °C were 29.0% and 20% for each incubation time.

Thus, S-layer protein increases the stability of liposomes at both evaluated temperatures.

3.2. The stability of S-layer liposomes was enhanced after glutaraldehyde treatment

Liposomes coated with native S-layer protein yielded one protein band with an expected molecular weight of 69 kDa for S-layer protein isolated from *L. kefir* and 49.5 kDa for S-layer protein isolated from *L. brevis* on a 12% SDS gel. The glutaraldehyde cross-linking of the S-layer lattice covering the liposomes was confirmed by the absence of the protein band corresponding to the S-layer protein subunits in the well of the SDS gel corresponding to 2% of glutaraldehyde (data not shown). The surface properties of the S-layer liposomes did not show changes as measured by the zeta potential, after the treatment of glutaraldehyde.

To evaluate of the effect of pH on liposome stability, calcein was used as the encapsulated media because the small variation of its fluorescence with the pH compared to CF. As shown in Table 1, liposomes coated with S-layer were able to retain more

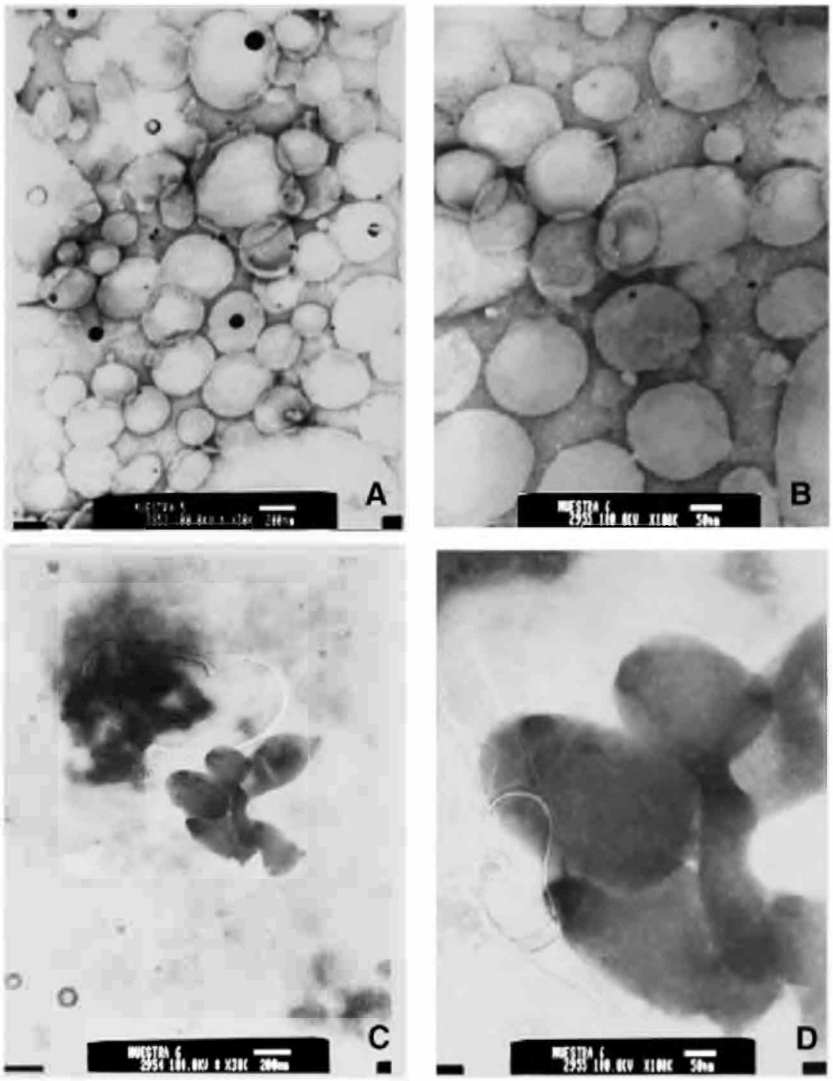

Fig. 5. Electron micrograph of a negatively-stained of control soybean lecithin liposomes (A and B) and prepared with 45 μg/ml of S-layer protein from *L. kefir* (C and D).

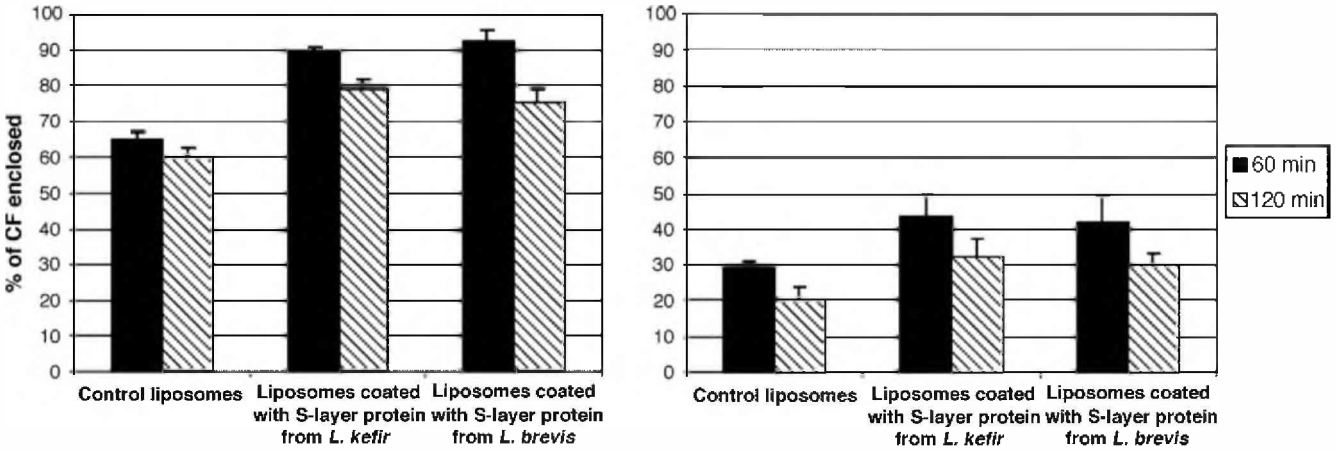

Fig. 6. Percentage of enclosed CF of liposomes prepared with 200 μg of S-layer from *L. brevis*, and 45 μg of *L. kefir* after incubation at 37 °C (A) and 50 °C (B) at two different times. Data represent the averages of three independent measurements from two batches of liposomes. Error bars indicate standard deviations of the means. The 100% of encapsulation was taken as that obtained by the addition of 10% Triton.

Table 1
Percentage of enclosed calcein after incubation of S-layer coated liposomes with and without glutaraldehyde treatment

	pH	60 min of incubation				120 min of incubation			
		Without glutaraldehyde treatment	SD	With glutaraldehyde treatment	SD	Without glutaraldehyde treatment	SD	With glutaraldehyde treatment	SD
Liposomes coated with	7	91.2%	5.2	94.1%	1.4	82.0%	2.9	91.3%	3.5
S-layer protein	4	57.3%	3.6	82.3%	4.3	49.2%	3.1	73.5%	3.1
from *L. brevis*	2.5	51.1%	4.5	74.5%	2.2	43.3%	4.3	65.9%	2.7
Liposomes coated with	7	89.5%	4.2	94.2%	5.3	86.5%	5.7	89.2%	1.8
S-layer protein	4	58.3%	2.3	73.6%	4.7	54.6%	5.1	65.2%	2.6
from *L. kefir*	2.5	51.2%	2.1	71.9%	2.1	49.4%	2.9	62.4%	1.7
Control liposomes	7	77.3%	1.7	54.4%	3.4	51.2%	1.8	48.6%	5.4
	4	49.5%	3.4	54.5%	4.1	33.1%	4.1	43.3%	3.8
	2.5	39.7%,	5.1	44.6%	2.8	13.3%	3.3	28.7%	5.1

calcein in comparison with control liposomes without S-layer in the different pH conditions evaluated (2.5, 4 and 7).

At pH 2.5, the values for control liposomes were 39.7% and 13.3% for 60 and 120 min. In contrast, 51.1% and 43.3% of retention was found after 60 and 120 min of incubation for liposomes coated with S-layer protein from *L. brevis*, respectively. For liposomes coated with S-layer protein from *L. kefir*, the retentions were 51.2% and 49.4% at each time of incubation, respectively.

At pH 7, liposomes coated with S-layer protein from *L. brevis* were able to retain 91.2% and 82.0% after incubation during 60 and 120 min, respectively and liposomes coated with S-layer protein from *L. kefir* retained 89.5% and 86.5%, respectively. In the same conditions, the values for control liposomes were 77.3% and 51.2%, respectively.

Finally, the percentages of calcein enclosed after incubation at pH 4 for 60 and 120 min for liposomes coated with S-layer from *L. brevis* were 57.3% and 49.2%, 58.3% and 54.6% for liposomes coated with S-layer from *L. kefir*, and 49.5% and 33.1% for control liposomes without S layer, respectively.

In the same Table 1, the results when S-layer-coated liposomes were treated with glutaraldehyde before the pH incubation are shown. The ability to retain the fluorescent probe increased in 20% for liposomes coated with S-layer proteins, in contrast to only 10% for control liposomes without S-layer.

With the aim to evaluate the stability of the vesicles in conditions mimicking the gastrointestinal media, the effect of bile salts and pancreatic extract was studied. After incubation times of 60 and 120 min with pancreatic extract, liposomes coated with S-layer protein were able to retain more CF than control liposomes. In liposomes coated with *L. kefir* S-layer the percentage of CF enclosed after incubation were 79.5 and 62%, respectively. These values were slightly lower in liposomes coated with S-layer from *L. brevis* (75.3 and 48%). Control liposomes showed values significantly lower at both incubation times, 20.0 and 12.0%, respectively (Fig. 7). The effect of incubation in bile salt after 60 and 120 min is shown in Fig. 8. Liposomes coated with S-layer protein were able to retain more CF than control liposomes. The percentages of CF enclosed by

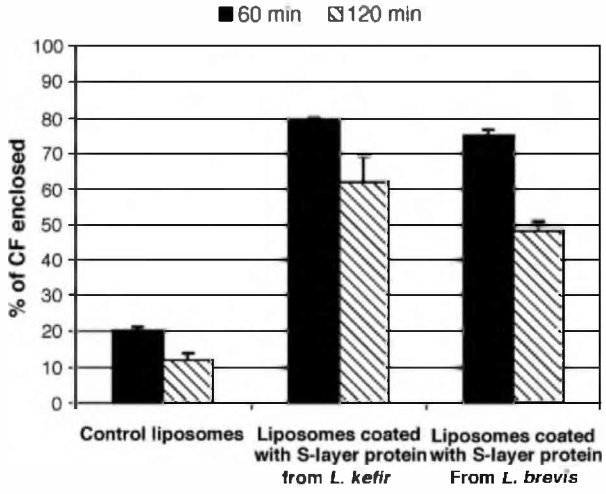

Fig. 7. Percentage of enclosed CF of liposomes prepared with 200 μg of S-layer from *L. brevis*, and 45 ig of *L. kefir* after incubation in pancreatic extract at 37 °C at two different times. Data represent the averages of three independent measurements from two batches of liposomes. Error bars indicate standard deviations of the means. The 100% of encapsulation was taken as that obtained by the addition of 10% Triton.

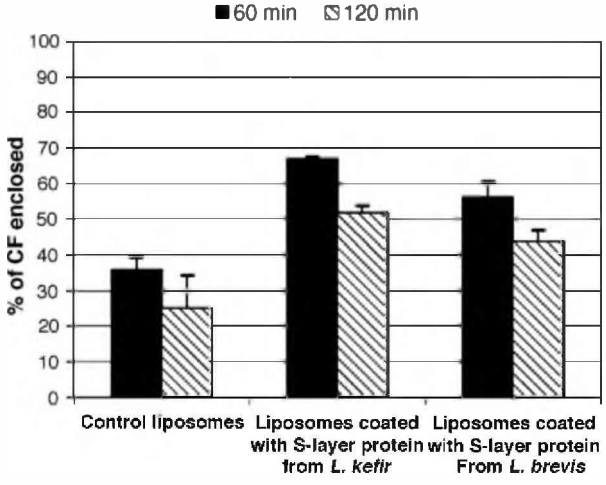

Fig. 8. Percentage of enclosed CF of liposomes prepared with 200 μg of S-layer from *L. brevis*, and 45 μg of *L. kefir* after incubation in bile salts at 37 °C at two different times. Data represent the averages of three independent measurements from two batches of liposomes. Error bars indicate standard deviations of the means. The 100% of encapsulation was taken as that obtained by the addition of 10% Triton.

liposomes coated with S-layer from *L. kefir* were 66.8 and 51.0% for 60 and 120 min of incubation, respectively. These values were slightly lower in liposomes coated with S-layer from *L. brevis* (56.1 and 43.6%). Control liposomes showed values significantly lower at the two incubation times, 35.8 and 25.2% respectively.

4. Discussion

This is the first study in which S-layer proteins isolated from lactobacilli species are used to cover liposomes. In this first study, the emphasis was given in testing the stability properties against biological media through which the liposomes must diffuse.

SA liposomes were used in order to design liposomes suitable lipid for therapeutics in humans substituting this component.

In the present work, it was observed that Z potential values of both liposome preparations decreases after the incubation with the S-layer proteins, in good agreement with the report for other S-layer proteins [11]. The Z potential measurements denote the existence of protein–lipid interactions, which are dependent of the incubation time, the source of the S-layer protein, the presence and quantity of self-assembly products. The electron micrograph confirms that S-layer proteins are adsorbed on liposome surface, covering the whole surface. Thus, the value of zeta potential for this lipid protein ratio appears related to the coverage of the liposome surface.

This denotes that important contributions to the interaction are the electrostatic charges. However, the effect of both types of protein on the decrease of the surface potential is much more pronounced when the liposomes are composed by SL. In this case, the membrane is in the fluid state in comparison to those of DPPC that are in the solid gel state at the temperature of measurement.

As the percentage of charges in both types of liposomes are the same, it would be expected that the electrical neutralization would be similar regardless the phase state. However, the SL vesicles acquire a net negative charge that may arise by a shielding effect of the S-layer proteins on the stearylamine and choline charges and to an exposure of negative charges of the protein itself.

In contrast, in vesicles in the gel state (DPPC) a partial neutralization of the positive charges is achieved since the minimal potential reached was around +30 mV. These data denote that the protein adsorption on the liposome surface depends on the phase state of the membrane. In addition, it suggests that other forces, besides the electrostatic ones, may contribute to the protein–membrane interaction.

Although the stability experiments do not show important differences between the S-layer proteins of *L. brevis* and *L. kefir*, the latter appears to have more affinity for SL surfaces. This is derived from the observation that the lowest surface potential is achieved at around 4 times lower amounts of *L. kefir* than with *L. brevis* S layer proteins. As SL membranes are in the fluid phase and those of DPPC in the gel phase at the working temperature, S-layer adsorption would be favored in fluid membrane congruent to previous reports [18,19].

Congruently, in Fig. 4, a lower amount of S-layer proteins from *L. kefir* is needed to attain the maximum of the retention of CF. Values shown in Fig. 3 suggest that the S-layer coating followed a two step process, in good agreement with the report for *in vitro* recrystallization process for S-layers from *Bacillaceae*, where it was shown that the assembly kinetics are multiphasic, with a rapid initial phase leading to oligomeric precursors and slow consecutive rearrangement steps, which finally lead to extended lattices [20]. In addition, kinetics of attachment of *L. kefir* S layer proteins is slightly more rapid than that for *L. brevis* (Fig. 3). Further studies should be done to correlate the stability properties with the surface structural arrangement.

When stability of liposomes was evaluated, the retention achieved after different incubation times at 37 °C and 50 °C were similar between liposomes coated with both types of S-layers proteins, and higher than control liposomas (Fig. 6).

The liposomal preparations were also challenged against different pHs. From Table 1, the most favorable conditions for encapsulation can be derived. It appears that at pH 7, *L. brevis* S layer is effective to retain CF during at most 60 min without glutaraldehyde treatment. However, for longer times, i.e. 120 min, the glutaraldehyde treatment introduces an important increase in the retention. At this pH, *L. kefir* seems to be more effective than *L. brevis* without glutaraldehyde.

At pH values mimicking gastrointestinal pH (2.5), liposomes coated with both S-layer proteins showed more stability than control liposomal preparation, but the glutaraldehyde treatment appears necessary for both S-layer coated liposomes to obtain a better stability, even for shorter times.

Some difference favors liposomes coated with *L. brevis* S-layer at pH 7. Although this suggests that the instability of the S-coated liposomes would be related to the permeation of protons, specific experiments should be done to address this question. The glycosylated S layer of *L. kefir* appears to reduce this process.

No striking differences were found with both proteins when S-layer-coated liposomes were challenged with pancreatic extract and bile salts. Although S-layers stabilize the lipid membrane against the detergent action, no influence of the glycosylated moieties of the *L. kefir* S-layer appears relevant.

The construction of stable and safe liposomal particles is an important issue for liposome technologies, in which the vesicles have to resist process steps like stirring, pumping or re-suspension, and transit across the gastrointestinal tract. Among the medical applications, increased stability of liposomal preparations will be essential in formulations which have to be administered by the oral route, in which liposomes will be exposed to harsh environment.

The development of drug targeting and delivery systems based on liposomes coated with functional S-layer isolated from beneficial bacteria such as lactobacilli appears in the light of the present results as a possibility to enhance their efficiency and stability.

In conclusion, S-layer proteins from both lactobacilli studied were able to coated stabilize liposomes against pancreatic extract, bile salt and thermal shocks, either the protein was glycosylated or not. In contrast, glycosylation appears to influence the stability of S-layer liposomes against pH gradients. The stability against pH gradient was moderately favored in *L. kefir* S-layer coated liposomes after the treatment with glutaraldehyde. Stabilizing effects observed in this work are in good agreement with the reported for other S-layer proteins [21,11].

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