

## S-layer proteins from lactobacilli as vaccine delivery systems



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**Abstract-** The S-layer, crystalline arrays of proteinaceous subunits, seems to be a typical surface structure in several lactobacilli species. Due their self-assembly ability to recrystallize into isoporous monolayers in suspension, at liquid-surface interfaces, lipid structures and on solid supports, S-layers were demonstrated to possess a great potential for nanobiotechnological applications. Interest in lactobacilli S-layer has been reinforced by claimed and demonstrated probiotic properties for human and animal consumers. Several lactobacillar S-layer have been found to be involved in adherence to intestinal epithelial cells and to the mammalian extracellular matrix. Due to these observed adhesive properties, the possible therapeutic applications of lactobacillar S-layers have become increasingly of interest, e.g. as targeted antigen delivery vehicles to host tissues. In addition, S-layers may provide superior expression levels and surface density of antigens when compared to other bacterial antigen presentation systems. It has already been demonstrated that S-layer protein subunits can be modified to carry foreign epitopes as a uniform recombinant S-layer on the *Lactobacillus* cell surface. The adhesion and immunogenic functions of S-layer proteins, combined with the properties of *Lactobacillus* spp., could lead to new, safe, and stable liposomal particles for drug delivery.

**Key Words:** S-layer proteins, lactobacilli, oral delivery, liposomes, vaccine design

### INTRODUCTION

Lactobacilli are Gram-positive, rod-shaped members of the lactic acid bacteria (LAB) group; they are characterized by the formation of lactic acid as the sole or main product of sugar fermentation. They are found where rich, carbohydrate-containing substances are available; hence LAB occur in a wide variety of habitats such as the mucosal membranes of humans and animals (oral cavity, vagina and gastrointestinal tract), on plants, in manure and man-made habitats such as sewage and fermented or spoiled food [1, 2]. The food and feed industry utilizes lactobacilli widely in different fermentative processes [3]. Due to their long history of use in food fermentation and in the food industry, and because of their lack of pathogenicity, lactobacilli are 'generally recognized as safe' (GRAS) organisms [4]. One of the outer surface components of cell envelopes of prokaryotic organisms, archaea and bacteria, are crystalline arrays of proteinaceous subunits, known as surface layers (S-layers), which usually are composed of single protein or glycoprotein species [5, 6, 7]. S-layer proteins have the capability to recrystallize into isoporous monolayers in suspension, at liquid-surface interfaces, lipid structures and on solid supports. Due their self-assembly ability, S-layers were demonstrated to possess a great potential for nanobiotechnological applications. The S-layer seems to be a typical surface structure in several lactobacilli species [4]. Interest in lactobacilli S-layer has been reinforced by claimed and demonstrated probiotic properties

for human and animal consumers [1, 8]. The aim of the present work is to organize the vast information concerning S-layer proteins, particularly S-layers from lactobacilli, spotlighting/underlining those aspects related to properties that make them attractive candidates for mucosal vaccine-delivery vehicles.

### S-layer proteins: Generalities

#### 1.1 Location and ultrastructure

When present, S-layers completely surround cells as the outermost component of their envelope, except for the organisms which possess capsule like *Bacillus anthracis*, where the capsule is the external structure [9]. They have been described in most of the main branches of the Bacteria and Archaea domains [9, 11, 12]. In archaea, S-layer lattices often constitute the only cell wall component, and some of them are anchored by pillar-like domains to the plasma membrane [13]. In Gram-positive bacteria, the lattice assembles on the surface of the cell wall, which is composed mainly of peptidoglycan.

In Gram-negative bacteria, the S-layer is attached to the lipopolysaccharide (LPS) component of the outer membrane [14]. Some Gram-positive and Gram-negative bacteria can produce two superimposed S-layers; usually, each is composed of a different subunit species [15], Fig. (1). Molecular masses of S-layer proteins range from 25 to 220 kDa; within species, S-layer proteins may also vary in size, depending on strain and growth conditions

[[16], [17]]. Most S-layers consist of a single protein or glycoprotein that polymerizes to form bidimensional paracrystalline structures. Only a few organisms, including *Clostridium difficile* and *B. anthracis*, have S-layer proteins with two types of subunits [[18], [19], [20]]. Electron microscopy of negative staining or freeze-etched preparations, Fig. (2), are necessary to identify S-layers [[15]].

High-resolution studies on the mass distribution of lattices are generally made with negatively stained, frozen S-layer fragments, or with recrystallized S-layer lattices. Surface topography of S-layers can also be studied through atomic force microscopy (AFM), which is increasingly used to investigate the forces within or between single biomolecules [[21]]. The S-layer crystal lattice may have oblique (p1, p2), tetragonal (p4), or hexagonal (p3, p6) symmetry. Depending on the pattern, a morphological unit of the S-layer consists of one, two, three, four, or six protein subunits, Fig. (3). Center-to-center spacing of the morphological units can vary from 2.5 to 35.0 nm; thus, the lattices can be quite porous, with pores occupying up to ~70% of their surface. The pores within an S-layer are of identical size (usually in the 2–8 nm range) and shape. Frequently, two or more distinct classes of pores are present. A common feature of S-layers is their smooth outer surface and more corrugated inner surface. Most S-layer subunits are weakly acidic proteins, and thus showed a charge-neutral outer and a net-negative inner face at a neutral pH [[16]].

### 1.2 Isolation and self-assembly

Interactions between S-layer subunits, as well as between the S-layer and the supporting envelope, can be disrupted in a reversible manner by cation substitution or by a high concentration of chaotropic agents [[22], [14]]. This demonstrates that individual S-layer subunits interact with each other and with the supporting cell envelope components through noncovalent bonding. Isolated S-layer subunits frequently maintain the ability to recrystallize into regular arrays in suspension or on surfaces after removal of the isolation agent. These arrays obtained in vitro exhibit structural features that are often indistinguishable from those found surrounding the cells in vivo. S-layers differ considerably in their susceptibility to disruption into monomeric subunits.

They are normally isolated from purified cell wall fragments by the addition of hydrogen-bond-breaking agents (urea or guanidine hydrochloride), detergents (at pH <4.0) or by cation substitution (e.g. Na<sup>+</sup> or Li<sup>+</sup> replacing Ca<sup>2+</sup>) [[14]]. Intersubunit bonds are stronger than those that bind the lattice to the underlying envelope layer; this property is a basic requirement for continuous recrystallization of the lattice during cell growth [[15], [24]].

### 1.3 Chemical characterization and primary structure

Most S-layer proteins include little or no sulfur-containing amino acids and have a high content of acidic and hydrophobic amino acids, lysine being the predominant basic amino acid [[4]]. They usually have an acidic isoelectric point, but there are a few exceptions, including S-layer proteins from lactobacilli and the archaea *Methanococcus*, which have high pH isoelectric points. A remarkable characteristic of many archaeal and some bacterial S-layers is their glycosylation. The glycan of *Halobacterium* S-glycoprotein consists of short, predominantly N-glycosidically-linked sulfonated heterosaccharides [[23]]. In contrast, glycans isolated from Bacillaceae S-layers are assemblies of identical, repeating units, with up to 150 monosaccharide residues, attached primarily by O-glycosidic linkages. These include novel linkage types, such as  $\beta$ -glucose→tyrosine,  $\beta$ -galactose→tyrosine and  $\beta$ -N-galactosamine→threonine/serine [[25]]. The genetic information for S-layer glycan biosynthesis is usually present in S-layer glycosylation (slg) gene clusters acting in concert with housekeeping genes [[26]]. Concerning an overall function of S-layer glycoproteins in non-pathogenic bacteria, it is conceivable that, by representing the outermost cell surface structure of a bacterium, they participate in diverse cell surface phenomena and, simultaneously, contribute to a high diversification potential of the bacterial cell surface, which may be advantageous in the competitive natural habitat [[26]]. Carbohydrates may provide also local protection against proteases through steric hindrance and consequently contribute to the stability of S-layers [[27]]. Due to the lack of suitable tools for genetic manipulation of bacterial S-layer glycosylation pathways, progress in the elucidation of the glycan biosynthesis mechanism, was limited to in vitro testing of individual enzymes from these pathways [[28]] and to heterologous carbohydrate-engineering approaches in the past [[29]]. Recently Zarschler et al. [[30]] have reported a successful genetic manipulation of bacterial S-layer protein glycosylation in vivo with promising applications in nanobiotechnology [[30]]. No homology has been found among S-layer protein sequences from phylogenetically unrelated organisms, suggesting strong selective pressure against their maintenance (through proteases, phages and the immune system). The main exception to this rule is the finding of one or more copies of the S-layer homology (SLH) motif in the S-layer sequences of some Gram-positive bacteria and of the *Thermus* group [[31]]. Tandemly organized SLH motifs have also been identified in extracellular enzymes from Gram-positive bacteria [[32]]; in these enzymes, SLH motifs facilitate binding to peptidoglycan [[27], [33]]. S-layer-peptidoglycan associations are mainly

mediated by specific contacts (SLH) domains either bind directly to the peptidoglycan [[34]] or to a pyruvylated carbohydrate that is covalently linked to the peptidoglycan [[25]].

## 2. S-layer proteins from lactobacilli

S-layers have been found on the cell surface of several Lactobacillus species of the *L. delbrueckii*, *L. reuteri*, *L. brevis*, *L. buchneri* and *L. casei* phylogenetic groups [[35], [1]], Fig (4). Lactobacillar S-layers have some common and distinctive features, such as a low content of cysteine and methionine, as well as a high content of hydrophobic and hydroxyl amino acids. Relatively small sizes (25 to 71 kDa) are characteristic of lactobacillar S-layer proteins [[4]]. These proteins also have an N-terminal secretion peptide (SP), as well as many other prokaryotic S-layer proteins [[12]]. Several members of the *L. delbrueckii* group possess two genes encoding surface proteins, one silent and one actively transcribed [[36], [37]] (Table 1). These bacteria may express alternative S-layer protein genes, probably to adapt to different stress factors, such as drastic changes in the environmental conditions. Generally, S-layer variation is a consequence of DNA rearrangements; however, variation of S-layer protein content in *L. brevis* ATCC 14869 occurs through a unique mechanism involving activation of transcription by a soluble factor, constituting response to changes in the environment [[17]]. Multiple genes coding for S-layer proteins is a feature that lactobacilli share with other bacteria, including *Bacillus anthracis* [[38]], *Clostridium fetus* [[39]] and *Tannerella forsythia* [[40]]. In S-layer proteins of lactobacilli, no SLH motifs have been detected but the attachment of the S-layer protein to the cell wall seems to involve also secondary cell wall polymers in several species [[27]]. The S-layer proteins from *L. brevis* and *L. buchneri* are reported to bind to a neutral polysaccharide moiety of the cell wall [[41], [42]]. Interaction studies performed with truncated rSlpA of *L. brevis* ATCC 8287 confirmed that N-terminal region of S-layer protein is responsible to cell wall binding [[43]]. On the other hand, the location of the cell wall binding domain of the *L. acidophilus* ATCC 4356 SlpA and *L. crispatus* JCM 5810 CbsA has been determined to reside in the C-terminal one third of these proteins. Interactions between a positively charged S-layer protein region and negatively charged secondary cell wall polymers have been shown to mediate the cell wall binding in the case of SlpA of *L. acidophilus* ATCC 4356 [[44]] and CbsA of *L. crispatus* JCM 5810 [[45]]. It was shown that SlpA and CbsA bind to teichoic acids, and CbsA binds also to lipoteichoic acids purified from *Staphylococcus aureus* and *Streptococcus faecalis*, but not to the teichuronic acid/polysaccharide fraction of the cell wall of *L. crispatus* JCM 5810 [45].

### S-layer glycoproteins in lactobacilli

Glycosylation represents the most common post-translational modification in S-layer proteins and it has been demonstrated in many archaeal and bacterial genera. Although most of S-layers from lactobacilli appear to be non-glycosylated [4] to date, glycan structures have only been found in *L. buchneri* [[46]], *L. kefir* [[47], [48]] and *L. helveticus* [[49]]. For *L. kefir* recently were described five molecules of glucose and five molecules of mannose [[47]] in a good correlation with previously reported from *L. buchneri*, which have six to eight molecules of glucose for each protein unit [[50]]. In both proteins, the glycan structures represent less than 10 % of weight, in concordance with data reported for other S-layer proteins describing that the degree of glycosylation generally varies between 1% and 10% (w/w) [[51]]. It was reported that glycosylation adds significantly to the potential functional spectrum of S-layer proteins [[51], [23]]. Mobili et al. [[48]] have been proposed that glycosylation on S-layer protein of *L. kefir* could be related with the aggregation ability of these bacteria. Furthermore, the presence of glycosidic groups on this bacterium seems to be related with a higher affinity of these proteins with positively charged lipid membranes [[53]]. However, further studies should be made in order to clarify the specific relationship between glycosylation and affinity features of S-layer proteins from *L. kefir*. Besides these effects, it has been reported that glycosylation contributes to protein antigenic properties [20], an interesting feature in the development of a vaccine carrier. Glycoengineering applications appears as a novel and interesting field of study that may include receptor mimics, vaccine design, or drug delivery using carbohydrate recognition [30]. Since the S-layer glycome is connected with a molecular self-assembly system, there is a strong link to the field of nanobiotechnology, because means for organizing materials, such as biologically functional glycans, at the nanometer level are prime candidates for the production of supramolecular structures and devices [[51]].

### 2.2. S-layer proteins functions

Synthesizing S-layer proteins is energy consuming and their productions are exquisitely regulated, suggesting that they each have a defined role [[9]]. However, no general function has been identified for S-layer proteins and many of the functions assigned are still hypothetical. Though they have been found in most of the phylogenetic branches of microorganisms, the function of S-layers is assumed specific for genera or groups of organisms in the same environment rather than common to all prokaryotes [[9]]. S-layers function as protective coatings, molecular-sieves in the ultrafiltration range (constituting molecule and ion traps), promoters of cell adhesion and surface recognition, determiners of cell shape in archaea and some bacteria,

and virulence factors in pathogenic microorganisms [[4]]. Little is known about the function of S-layer proteins in lactobacilli. They have been found to be involved in adherence to intestinal epithelial cells [[54], [55]] and to the mammalian extracellular matrix [[56], [37], [57]] (Table 2). *L. crispatus* JCM 5810 adheres efficiently to collagen and laminin, which are major components of the mammalian extracellular matrix (ECM); extracted S-layer protein from this species binds to collagen IV [[57]]. The N-terminal portion of the molecule is responsible for binding to host receptors [[45]]. This was also observed for S-layer proteins Slp A of *L. brevis* ATCC 8287 [[56]]. Jakava-Viljanen et al. found that S-layers of lactobacilli isolates from pig intestines and feces adhere to mammalian intestinal-epithelial-cell lines, intestinal and gastric mucus, and ECM components [[58]]. Treatment of *L. kefir* and *L. parakefir* cells with lithium chloride (LiCl) eliminate their hemagglutination properties [[22]]. Adhesive functions were also suggested for S-layer proteins from *L. acidophilus* M92 [[51]]. Adherence of probiotics to intestinal epithelial cells and temporary colonization of the gut are considered crucial for their beneficial health effects [[59], [60]]. Adhesive S-layers can inhibit adhesion of pathogenic bacteria, contributing to the probiotic effects of lactobacilli. Probiotic activity is generally a function of bacterial surface properties [[61], [62]]. Recently, based on analysis by surface plasmon resonance, SlpA of *L. brevis* ATCC 8287 was found to interact with fibronectin and laminin [[63]]. Good adhesion properties could play a role in the competitive exclusion of potentially harmful microbes by *L. brevis* ATCC 8287. Golowczyk et al. [[64]] showed that preincubation of salmonella cells with S-layer proteins from *L. kefir* leads to changes in the surface properties of this bacterium, so that they are no longer able to invade cultured human enterocytes. That S-layer protein from *L. kefir* is also responsible of co-aggregation with the yeast *Sacharomyces lipolitica*. These results and the capacity of purified S-layer proteins of *L. kefir* to haemagglutinate strongly suggest that a lectin-like activity of bacterial surface proteins (S-layer) mediates the aggregation with yeast cells [[65]]. The S-layer proteins of *L. crispatus* strain ZJ001 is responsible for competitive exclusion against *Escherichia coli* O157:H7 and *Salmonella typhimurium* [[66]]. In addition, S-layer protein extracts from *L. helveticus* R0052 can inhibit enterohaemorrhagic *E. coli* adhesion to host epithelial cells [[67]]. Zhang et al. [[68]] have shown that *L. paracasei* subsp. *paracasei*, *L. rhamnosus*, and *L. casei* strains isolated from natural dairy products are capable of exerting a significant degree of inhibition of *Shigella sonnei* adhesion to HT-29 cells, and their S-layer proteins contributed to this inhibition activity. For S-layer protein of *L. acidophilus* ATCC 4365 was described a true murein hydrolase activity associated with this S-layer,

with lytic activity toward the cell walls of several bacteria [[69]]. A role of murein hydrolase for an S-layer protein had previously been described only for *Bacillus anthracis* [[69]]. Furthermore was also probed that, in combination with nisin, the S-layer from *L. acidophilus* acts synergistically to inhibit the growth of pathogenic *Salmonella enteric*, *Staphylococcus aureus* and *Bacillus cereus* [[70]]. Recently, the obtention of knockout for S-layer protein SlpA in *L. acidophilus* allowed a better understood of S-layer proteins; the slpA mutant grew as small curved bacilli, reinforced the idea of S-layer proteins are involved in cell shape determination [[54]]. The slpA mutant also lost in 84% its adhesive properties to Caco-2 cells. Removal or alteration of a surface layer can have many dramatic cellular effects, not the least of which may be on surface charge, architecture, and the presence or conformation of various surface proteins involved in attachment [[54]]. Other functions for S-layers have also been identified. The S-layer protein of *L. helveticus* CNRZ 892 functions as a receptor for a phage [[71]]. S-layers have been proposed to have a role in cell shape determination and cell wall stabilization [[15]]. Extraction of S-layer proteins reduced the viability of *L. acidophilus* in simulated gastric and small intestinal juice, suggesting a protective role for the S-layer [[71]].

Taking into account all above-mentioned S-layer functions that reinforce the probiotic properties of lactobacilli, characterization of different lactobacillar S-layer proteins is key to the selection of useful strains.

#### 4. S-layer proteins in vaccine technology

##### 4.1 S-layer proteins as immunogenic agents

One of the most relevant areas of research in nanobiotechnology is the technological utilization of self-assembly systems [[72]]. Individual S-layer subunits contain all the information required for assembly into a regular array; isolated subunits can assemble into organized structures, resembling those observed on intact cells [[15]]. The natural assembly of S-layers into large regular arrays endows them with immune-stimulating and intrinsic-adjuvant properties [[51], [73]]. The location of the foreign antigen within the S-layer protein may not be crucial for vaccine production, as long as sufficient amounts of expression are obtained and sheet-like structures are formed. The potential of S-layers as antigen carriers for vaccine preparations has been demonstrated [[74], [75]]. Polysaccharides and proteins can be chemically linked to S-layers; the use of such conjugates induces immune responses in animal models. Insertion of foreign proteins into S-layers by genetic manipulation avoids the need for toxic chemicals and ensures the presence of these proteins in every S-layer protein subunit [[76]]. T-cell-mediated immunity can be induced in BALB/c mice by

oligosaccharide haptens immobilized on crystalline S-layers [[72]]. The use of S-layer proteins as a carrier for birch pollen allergen diverts the response to vaccination from an allergic TH2-mediated reaction towards a more favorable TH0/TH1 reaction [[75]]. High levels of recombinant S-layer protein expression have been achieved with bacterial ghosts. A combination of recombinant S-layers and bacterial ghosts is one of the potential applications of ghost-platform technology. Sheet-like S-layer structures, harboring the desired antigen, are entrapped in the cytoplasm of the ghost during the lysis process [[77]]. The sealed periplasm of a ghost constitutes another compartment that can be filled with soluble proteins [[77]] or recombinant S-layers [[76]]. Whether it is advantageous to present the antigen in different compartments remains to be elucidated [[76]]. Immunization with purified Omp26 S-layer protein from *Bacillus thuringiensis* was found to be suitable for cell-surface display [[78]]. This approach opens the possibility of generating live, heat-stable, oral veterinary vaccines against infectious diseases of fowl, using the *Bacillus thuringiensis* S-layer protein CTC surface-display system [[79]]. Recently Khang et al. [[80]] showed that a mixture of the *L. brevis* S-layer fusion proteins and the specific antibodies against coronavirus, rotavirus, *Escherichia coli*, and *Salmonella typhimurium* to newborn Hanwoo calves twice a day resulted in successful protection from neonatal calf diarrhea syndrome for about three weeks. This is the first report to treat neonatal calf diarrhea syndrome effectively using the recombinant Lactobacillus S-layer proteins. Thus, the results open promising applications of the bacterial S-layer proteins in the animal industry as well as in the bioindustry for protein immobilization [[80]]. Konstantinov et al. [[81]] recently established that the SlpA-dominant *L. acidophilus* NCFM interacts with a major receptor on dendritic cells and regulates its immune functions. It suggests that this probiotic bacterium could directly or indirectly interfere with pathogen-induced effects on the host immune system. These data establish a working hypothesis on the mode of action of probiotic cultures that will guide further investigations into the mechanisms by which these bacteria impact GI states. The work also suggests that the potential switch between S-layer proteins SlpA and SlpB might lead to differential immune responses impacting the gut immune homeostasis. Therefore, further studies need to address at what extent the ratio of SlpA to SlpB present on the surface of different intestinal LAB [[81]].

#### 4.2 Lactobacillar S-layer proteins and vaccine delivery

Lactobacilli have several properties that make them attractive candidates for mucosal vaccine-delivery vehicles. The intrinsic properties of lactobacilli to modulate the

immune system make them attractive for health applications, in particular for the in vivo production and delivery of biologically active molecules [[82]]. Modulation of the immune system can occur through the intrinsic adjuvanticity and cytokine-inducing properties of lactobacilli. Recently, there has been increased interest in the possibility of genetically manipulating lactobacilli, both for probiotic use [[83]] and for delivering mucosal vaccines [[84]]. Various cellular locations have been tested for antigen production with lactococci and lactobacilli [[85], [86]]. If Lactobacillus species could be engineered in such a way that each S-layer protein monomer was fused to a foreign antigen, the concentration of antigen at the bacterial surface would be very high. Identification and characterization of adherence factors has therefore become a topic of special interest [[87]]. S-layer proteins can account for 10–12% of total cell proteins. This high level of expression facilitates large-scale production of target proteins. More importantly, since S-layer proteins are expressed on the cell surface and are either secreted or can be easily released from the cell surface, recovery and purification of S-layer fusion proteins is relatively simple [[88]]. Smit et al. [[44]] demonstrated that it is possible to surface-expose epitopes as part of the S-layer of *L. acidophilus*. Efficient transcription of S-layer protein genes is required, since synthesis of up to  $5 \times 10^5$  S-layer protein subunits has been calculated to be required for complete formation of an S-layer on an average-sized cell [[89]]. Studies in the activities of the two promoters of SlpA from *L. brevis*, by measuring reporter enzyme activities and transcript amounts in recombinant strains, showed the high expression levels of both slpA and the reporter genes. Furthermore, recombinant *L. brevis* strains remain on a substantially high level under conditions encountered in the gastrointestinal tract, by addition of bile or pancreatic extract in the growth medium, or after a change the carbon source. These findings encourage the research aiming at the use of *L. brevis* ATCC 8287 in live oral vaccines with its S-layer protein as an antigen carrier [[90]]. Similar results were found for slpA gene of *L. acidophilus* ATCC 4356, where slpA gene expression increased in the salt bile stress conditions [[91]]. Furthermore, in S-layer proteins of *L. brevis* ATCC 8287 it was demonstrated that self-assembly domain comprises residues 179–435 in mature protein, since truncated proteins encompassing this region were able to form a periodic structure indistinguishable from that formed by full length SlpA. The mapping of surface exposed residues in the self-assembly domain of SlpA is currently in progress and is a potential tool for mucosal immunization, and further studies concerning vaccine development [92]. Small epitopes can be surface-displayed as part of the S-layer proteins of lactobacilli [[92], [44]].

An intact S-layer without any changes in crystalline structure can be formed even if there is a heterologous epitope in every S-layer subunit [[92]]. A poliovirus VP1 epitope and a c-Myc epitope from the human c-myc proto-oncogene have been successfully surface displayed in *L. brevis* SlpA protein [[92], [44]]. Recombinant *L. brevis* that displayed the c-Myc epitope in all of the S-layer protein subunits, without any effect on S-layer lattice structure, was obtained because of successful gene replacement [[92]]. Surface display in S-layers would thus be a very efficient and safe means to present antigens to the mucosal-associated lymphoreticular tissue [[92]].

Recently was showed that both slpA and slpB S-layer genes of *L. crispatus* ZJ001 expressed in *E. coli* with the addition of His tags, were adhesive to HeLa cells, although different in the structure of protein polymers. Therefore, the authors suggest that it is possible to explore the strain *L. crispatus* ZJ001 as a mucosal vaccine delivery vector, by engineering heterologous protein genes into slpA, because of the expression pattern and high cell binding ability of SlpA [[93]].

#### 4.3 Liposomes stabilized by S-layer proteins as vaccine carriers

Liposome-antigen and liposome-DNA vaccines have frequently been tested using systemic routes of administration; various new products based on this technology are presently in use or in clinical development [[94]]. Biodegradability and low permeability to small hydrophilic molecules make liposomes excellent reservoirs for drug loading/release [[95]]. Development of effective delivery systems for the presentation of antigens to mucosal surfaces is critical to the success of vaccines [[96]]. Particulate antigens are more effective than soluble antigens for the induction of systemic and mucosal immunity, possibly because of more efficient endocytosis of particulate antigens by mucosal-associated lymphoid tissue (MALT) [[97]]. Liposomes are considered effective immunological adjuvants and have potential for intranasal and oral delivery of protein antigens [[98]]. However, delivery of pharmaceuticals via an oral route, particularly biotechnology products such as proteins, peptides, genes, oligonucleotides and vaccines, remains problematic to this day. Instability in the gastrointestinal environment and poor permeability across the intestinal epithelial cell barrier contribute to the poor oral bioavailability of many of these compounds [[99]]. Modulating the properties of vesicles by means of covalently or non-covalently attached or entrapped polymers [[100]] helps control their stability and permeability and imparts some specific surface properties [[95]]. S-layer proteins of some *Bacillus* and *Lactobacillus* species can be crystallized on liposomes composed of various phospholipids together with cholesterol, leading to a novel type of liposome (Table 3). Such S-layer coated

liposomes are closed biomimetic structures that resemble archaeal cells, in which the S-layer proteins crystallize over the plasma membrane [[101]]. Natural association of S-layers with the cytoplasmic membrane occurs only in Archaea, where S-layers are anchored by hydrophobic stretches of protein 'stalks' that presumably act as immobilization structures for lipids and proteins, with unknown consequences for membrane properties in vivo [[13], [102]]. Interaction of S-layer proteins with lipid molecules is noncovalent. Electrostatic interaction between exposed carboxy groups on the inner face of the S-layer lattice and zwitterionic lipid head groups is primarily responsible for the binding and defined orientation of the S-layer subunits to form a closed lattice structure. It has been suggested that there are at least two to three contact points between the lipid film and the attached S-layer protein. Therefore, only a few lipid molecules would be anchored via their head groups to protein domains on the S-layer lattice; the remaining scores of lipid molecules diffuse freely in the membrane between the pillars consisting of anchored lipid molecules. Because of its fluid characteristic, this nano-patterned type of lipid membrane is also referred to as a 'semifluid membrane' [[103]]. However, most importantly, the attached S-layer lattices apparently have no effect on the hydrophobic lipid acyl chains. Thus, S-layer lattices constitute unique scaffolding for lipid membranes [[104]]. Use of these properties has potential for a broad spectrum of developments in many areas, including diagnostics, high-throughput screening for drug discovery, sensor technology, and electronic or optical devices; it might find application in DNA sequencing [[101]]. S-layer proteins, after they have been crystallized on liposomes, can be cross-linked and exploited as a matrix for the covalent attachment of functional molecules, which would be useful for drug targeting or immunodiagnostic assays [[106], [107]]. Although currently the most extensively studied S-layer proteins are those of *Geobacillus stearothermophilus* PV72/p2 and *Bacillus sphaericus* CCM 2177, many relevant nanobiotechnological applications can be expected from the utilization of S-layer proteins from other organisms, in particular *Lactobacillus* [[7]]. In recent studies with S-layer proteins of lactobacilli, we demonstrated the existence of protein-liposome interactions. Using electron micrographs, we showed that S-layer proteins are adsorbed on the liposome layer, covering the entire surface [[109]]. Furthermore, S-layer proteins isolated from GRAS lactobacilli are able to stabilize positively charged liposomes against different condition [45]. This effect could be explained by the concurrence of different changes promoted by the proteins on the lipid bilayer. An increase in the bilayer packing due to charge neutralization decreases in the acyl chain mobility and hence permeability. In addition, S-layer proteins were

able to decrease liposome fusion, probably to the inclusion of bulky moieties protruding from the membrane, giving place to steric hindrance for surface-surface interaction [[53]]. Studies on Caco-2 monolayers using liposomes coated with S-layer proteins from *L. kefir* showed absence of cytotoxicity and also that coated liposomes were able to transfer more fluorescent molecules than control ones (without S-layer proteins) [[110]]. The natural adhesive properties of S-layer proteins to different epithelial cells probably promote the internalization, could explaining those results. The higher stability of S-layer-coated liposomes, combined with the adhesion and immunogenic functions of S-layer proteins, and the possibility for immobilizing or entrapping biologically active molecules [[106]] reveal a broad application potential, particularly as carrier and/or drug delivery [[103]]. S-layer lattices constitute unique supporting scaffoldings for lipid membranes that can be occupied by a great variety of membrane-active peptides and by peripheral and integral membrane proteins [[111]].

### 5. Conclusions and perspectives

S-layers have been identified in several *Lactobacillus* species. In some of these bacteria, S-layers have been shown to function as adhesins, mediating binding of lactobacilli to the host epithelial cells and/or ECM [[4]]. Due to these observed adhesive properties, including their high degree of structural regularity and their self-assembly properties, the possible therapeutic applications of lactobacillar S-layers have become increasingly of interest, e.g. as targeted antigen delivery vehicles to host tissues [[86], [111], [107]]. S-layers may provide superior expression levels and surface density of antigens when compared to other bacterial antigen presentation systems. It has already been demonstrated that S-layer protein subunits can be modified to carry foreign epitopes as a uniform recombinant S-layer on the lactobacilli cell surface [[92], [44]]. The application of the intrinsic adjuvanticity of lactobacilli as hosts, in constructing these vaccine strains, may result in the stimulation of immune responses to a co-administered antigen, surface-displayed as a part of the S-layer. Further advantages in employing lactobacilli as surface-display vectors include the health-promoting properties claimed for several strains [[112]], the GRAS status of lactobacilli [[113]] and the lack of LPS in the cell wall, which eliminates the risk of endotoxic shock [[84]]. However, there have been few immunization studies with *Lactobacillus* S-layer antigen constructs; favorable results from several immunization studies using recombinant S-layer proteins from other bacteria [[76], [88]] encouraged us to develop *Lactobacillus* S-layer based antigen carriers. The adhesion and immunogenic functions of S-layer proteins, combined with the properties of *Lactobacillus* spp., could lead to

new, safe, and stable liposomal particles for drug delivery. The high mechanical and thermal stability of S-layer-coated liposomes and the possibility for immobilizing or entrapping biologically active molecules [[114]] are important properties for liposome processing, as the vesicles have to resist processing manipulations, including stirring, pumping and resuspension, and they need to pass across the gastrointestinal tract. For medical applications, increased stability of liposomal preparations is essential for formulations that must be administered orally, because the liposomes will be exposed to a harsh environment [[109]]. Another potential application for lactobacillar S-layers is in nanotechnology, in which self-assembly systems are exploited for the activation of surfaces as well as for the production of biomimetic membranes and encapsulation systems [[7]].

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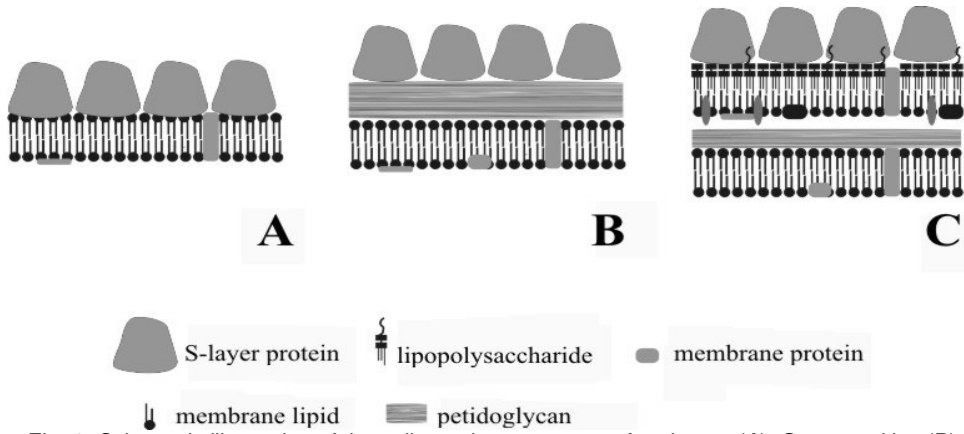


Fig. 1- Schematic illustration of the cell-envelope structure of archaeas (A), Gram-positive (B) and Gram-negative bacteria (C) with S-layer. Modified from Schuster et al. [[16]].

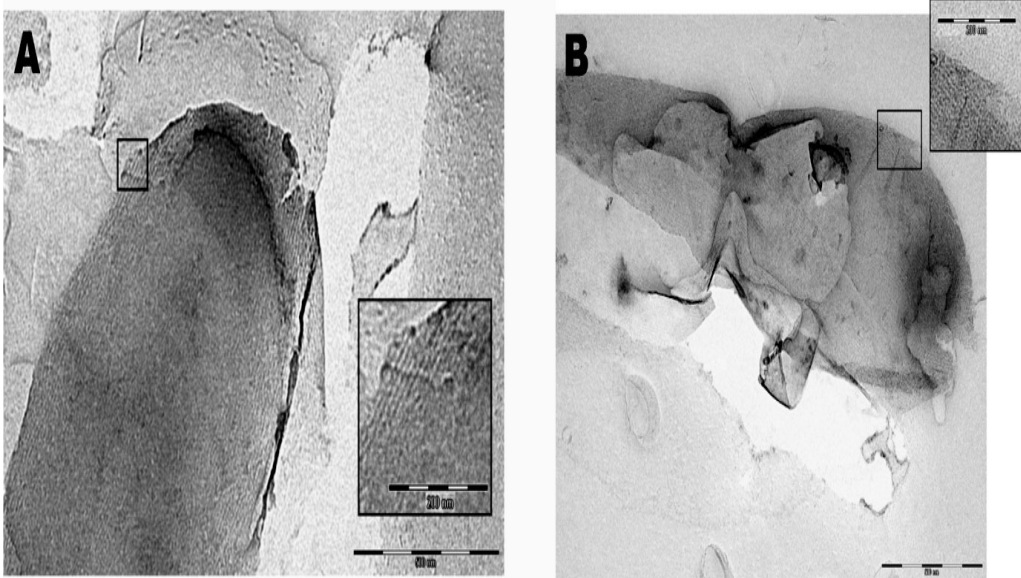


Fig. 2- Transmission electron micrographs of freeze drying entire cell of *L. kefir* (A) and *L. buchneri* (B). Barrs. 500 nm., Zoom in 200 nm.

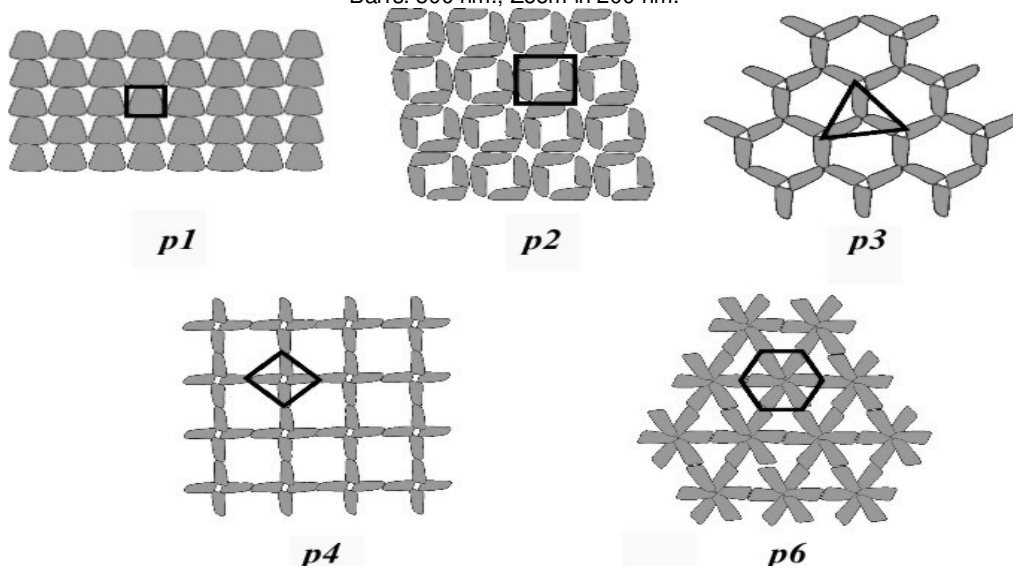


Fig. 3- Schematic drawings of the different S-layer lattice types. The regular arrays either show oblique (p1, p2), square (p4) or hexagonal (p3, p6) lattice symmetry. Modified from Sleytr et al. [[14]].

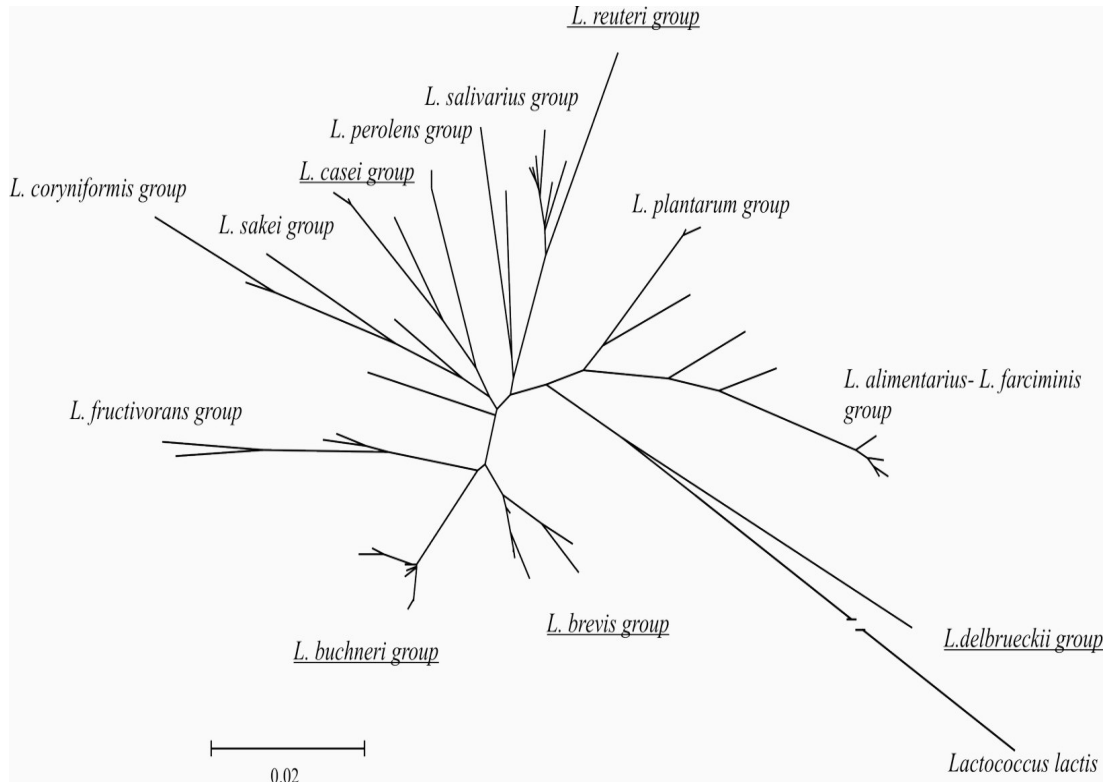


Fig. 4- Neighbor-Joining tree based on 16S rRNA gene sequences of Lactobacillus species defined by Fellis and Dellaglio, [115]. In bold underlined are indicated phylogenetic groups where S-layer carrying species have been reported.

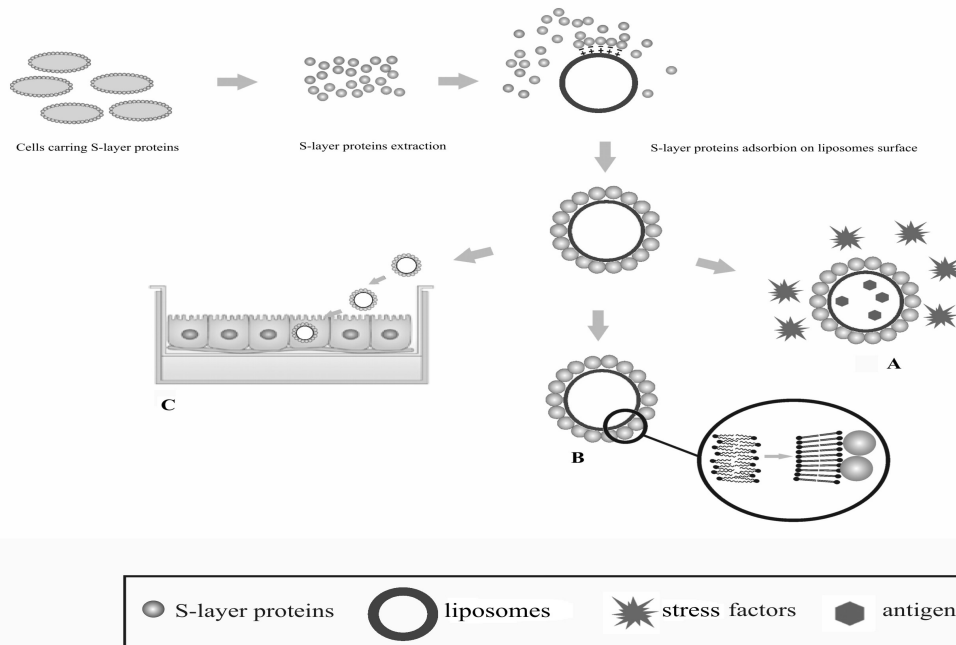


Fig. 5- Schematic representation of the S-layer protein obtention and adsorption to the external surface of liposomes and its effects against stress factors (A), on the lipid properties (B) and on their internalization rate on Caco-2 cells monolayers (C).

Table 1- *Lactobacillus* species that possess an S-layer

Species	Strain	Genes	Reference
<i>Lactobacillus acidophilus</i>	ATCC 4355		[116]
	ATCC 4356	<i>slpA, slpB</i>	[117, 36]
<i>Lactobacillus amylovorus</i>	LMG 9496	-	[118]
	7 isolates		[58]
<i>Lactobacillus brevis</i>	ATCC 8287	<i>slpA</i>	[119]
	ATCC 14869	<i>slpB, slpC, slpD</i>	[17]
<i>Lactobacillus buchneri</i>	ATCC 4005	-	[8]
<i>Lactobacillus casei</i>	ATCC 7469	-	[120]
<i>Lactobacillus crispatus</i>	JCM 5810	<i>cbsA, cbsB</i>	[57]
<i>Lactobacillus fermentum</i>	NCTC 7230	-	[42]
	NCIB 6991		
<i>Lactobacillus gallinarum</i>	ATCC 33199	<i>lgsB, lgsI</i>	[118]
	8 strains	<i>lgsA, lgsC, lgsD, lgsE, lgsF, lgsG, lgsH,</i>	[121]
<i>Lactobacillus gasseri</i>	ATCC 19992	<i>apf1, apf2</i>	[122]
<i>Lactobacillus johnsonii</i>	5 strains	-	[122]
<i>Lactobacillus helveticus</i>	ATCC 12046		[123, 124]
	CNRZ 892 (ATCC 10386)	<i>slpH1, slpH2</i>	
<i>Lactobacillus kefir</i>	20 isolates, JCM 5818, ATCC 8007	-	[22]
<i>Lactobacillus parakefir</i>		-	[22]

Table 2- Proposed or identified adhesive surface proteins of *Lactobacillus*

S-layer protein	Target	Species / Strain	Reference
S-layer protein	Avian intestinal epithelial cells	<i>Lactobacillus acidophilus</i> spp.	[55]
CbSA	Collagens, laminin	<i>Lactobacillus crispatus</i> JCM5810	[57]
SlpA	Fibronectin, human epithelial cell line	<i>Lactobacillus brevis</i> ATCC8287	[56]
S-layer protein	Red blood cells	<i>Lactobacillus kefir</i> CIDCA8321	[22]
		<i>Lactobacillus parakefir</i> CIDCA8328	
SlpA	Murine ileal epithelial cells	<i>Lactobacillus acidophilus</i> M92	[54]
S-layer protein	human epithelial cells	<i>Lactobacillus helveticus</i> R0052	[53]
S-layer protein	Mammalian epithelial cell	<i>Lactobacillus amylovorus</i> spp.	[58]
S-layer protein	<i>Sacch. lipolytica</i>	<i>Lactobacillus kefir</i> CIDCA 8315	[65]

Table 3- Evidence of S-layer proteins able to recrystallise on lipid interfaces

Organism	Lipid interface	Reference
<i>Lactobacillus brevis</i>	liposomes	[53, 109]
<i>Lactobacillus kefir</i>	liposomes	[53, 109]
<i>Bacillus sphaericus</i>	lipid monolayer	[128,129]
<i>Geobacillus stearothermophilus</i>	liposomes	[125]
<i>Bacillus coagulans</i>	liposomes	[127]

Table 4-Characteristics of liposomes coated with S-layer proteins.

Effect on liposome	Reference
Stabilization effects against	
Bile salt	[109]
Pancreatic extracts	[109]
Lower pH	[109]
Mechanical stress	[125]
Temperature stress	[109, 125]
Inversion of the $\xi$ -potential in positively charged liposomes	[109, 125]
Stabilization of the liquid-ordered gel-like state	[126]
Ordering of the lipid molecules	[53]
Inhibition of spontaneous fusion	[53]