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# INSIGHTS INTO INVOLVEMENT OF S-LAYER PROTEINS OF PROBIOTIC LACTOBACILLI IN RELATION TO GUT HEALTH

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**Abstract:** Probiotic lactobacilli possess outer cell wall structures composed of macromolecular paracrystalline arrays of proteins or glycoproteins known as surface layer proteins (S-layer proteins) for maintaining cellular structural integrity and conferring protection to the cells. Subunits of S-layer proteins are linked to each other and to the underlying cell surface by non-covalent forces. The presence of S-layer has now been described in several species of lactobacilli such as *Lactobacillus acidophilus, L. helveticus, L. casei, L. brevis, L. buchneri, L. fermentum, L. bulgaricus, L. plantarum, L. crispatus, L. kefir and L. parakefir* which range from 25 to 71 kDa. S-layer proteins consist of two functional domains *i.e.* the self-assembly domain and cell wall targeting domain. The interaction between *Lactobacillus* surface layer protein (S-layer protein) and gastro-intestinal epithelial cells activates various signaling pathways for conferring protection to the host by competitively blocking the adhesion of high risk pathogenic bacteria such as enteropathogenic *E. coli*, *Salmonella typhi*, *Shigella dysenteriae* and *Listeria monocytogenes* etc. Some of the probioticproperties such as adhesion, aggregation or pathogen inhibition have been related with the presence of S-layers. The S-layer protein from *L. crispatus* was found to prevent the adhesion of potential pathogen like *E. coli* 0175:H7 upon co-incubation of *E. coli* O157:H7 or *Salmonella typhimurium* with S-layer proteins from *L. crispatus* ZJ001 which prevented the adhesion of the pathogens to HeLa cells through a competitive exclusion mechanism. Besides their application in prevention of GI tract disorders, they have several other applications such as exploring them as nanoparticles due to the self-assembly nature of native and recombinant protein in suspension.

**Keywords:** Adhesion; Immunomodulation; Pathogen exclusion; Probiotics; S-layer Protein.
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## INTRODUCTION

Among lactic acid bacteria (LAB), many *Lactobacillus* strains have been characterized as probiotics. Probiotic lactobacilli possess outer cell wall structures composed of macromolecular para-crystalline arrays of proteins or glycoproteins known as surface layer proteins (S-layer proteins) for maintaining cellular structural integrity and conferring protection to the cells. S-layer proteins represent 10–15% of the total protein of the bacterial cell and the subunits of S-layer proteins are linked to each other and to the underlying cell surface by non-covalent forces. The presence of S-layer has now been described in several species of lactobacilli such as *Lactobacillus acidophilus, L. helveticus, L. casei, L. brevis, L. buchneri, L. fermentum, L. bulgaricus, L. plantarum, L. crispatus, L. kefir and L. parakefir* with size ranging from 25 to 71 kDa. The thickness of S-layer is about 5–25 nm and 70% area of the S-layer is occupied by porous structure. The pore size of S-layer varies from 2 to 8 nm (Avall-Jaaskelainen *et al.,* 2005; Mobili *et al.,* 2010; Sun *et al.,* 2013; Gerbino *et al*., 2015; Johnson *et al.,* 2016;Zhu *et al.,* 2017; Cavallero *et al.,* 2017). The amino acid composition, secondary structure, and the physical properties of these proteins were found to be quite similar in lactobacilli (Wasko

*et al.,* 2014; Hollmann *et al.,* 2017). S-layer proteins consisting of two functional domains *i.e*. the self-assembly domain and cell wall targeting domain which have been well characterized in *L. crispatus*, *L. acidophilus* and *L. brevis*. Eslamia *et al.* (2013) reported that S-layer protein from *L. acidophilus* ATCC4356 is stable in harsh conditions of gastrointestinal system such as simulated gastric fluid (SGF) with pH 2 up to 5 min. with and without pepsin and it is also stable in all the simulated intestinal fluids. It opens interesting perspectives in using and development of this S-layer as a protective coat for oral administration of unstable drug nanocarriers. Stuknyte *et al.*(2014) showed that isolated Single-chain variable-fragment antibodies (scFvs) is promising for the development of a rapid and accurate ELISA- based detection assay for the *L. helveticu s*MIMLh5 S-layer protein to characterize the potential immunomodulatory properties of dairy based foods.The interaction between *Lactobacillus* surface layer protein (S-layer protein) and gastro-intestinal epithelial cells activates various signaling pathways for conferring protection to the host by competitively blocking the adhesion of high risk pathogenic bacteria such as enteropathogenic *E. coli* particularly *E. coli* O157:H7, *Salmonella typhi*, *Shigella dysenteriae* and *Listeria monocytogenes* etc. (Johnson-Henry *et al.,* 2007; Zhang *et al.,* 2010; Cavallero *et al.,* 2017). Several key physiological and structural functions such as protective coat against many hostile factors, maintenance of cell shape, adhesion to mucus, extracellular matrix proteins (ECM), and epithelial cells, molecular sieve, interaction with immune cells and regulation of their function through cytokine induction and pathogen exclusion have been assigned to the S- layer (Jafarei *et al.,* 2011; Carasi *et al.,* 2014; Uroic *et al.,* 2016; Hymes *et al.,* 2016). However, the functions of S-layer proteins are highly species and strain specific since some of the probiotic properties such as adhesion, aggregation or pathogen inhibition have been related with the presence of S-layers. The S-layer protein from *L. crispatus* was found to prevent the adhesion of potential pathogen like *E. coli* O175:H7 (Chen *et al.,* 2007). Co-incubation of *E. coli* O157:H7 or *S. typhimurium* with S-layer proteins from *L. crispatus* ZJ001, prevented the adhesion of the pathogens to HeLa cells through a competitive exclusion mechanism.Xue *et al.,* (2014) also studied the role of S-layer proteins in increased adhesion and competitive exclusion of pathogens. SlpA of *L. acidophilus* NCFM was the first probiotic bacterial DC-SIGN ligand identified which is functionally involved in the modulation of dentritic cells and T-cells functions (Konstantinov *et al.,* 2008). Recently, Li *et al.* (2011a) have reported that S-layer protein of *L. acidophilus* inhibits apoptosis in Caco-2 cells induced by *S. typhimurium.* Kontroa *et al.* (2014) reported that Small Angle X-ray scattering (SAXS) and zeta potential measurements are useful techniques for characterizing the reassembly properties of crystalline structure formed by S-layer protein SlpA of *L. brevis* ATCC 8287 on positively charged liposomes. Khang *et al.* (2009) found that S-layer protein also plays an important role in alleviation of calf diarrohea. Besides their application in prevention of GI tract disorders, they have several other applications such as exploring them as nanoparticles due to the self-assembly nature of the native and recombinant protein in suspension. They also act as isoporous ultrafiltration membranes; hence can be used a carrier for vaccine development or as matrix for the immobilization of functional molecules or as supporting structure for functional lipid membranes etc.

**Probiotics**

Dr. Elie Metchnikoff, a Russian laureate, is credited with introducing the concept that live microorganisms are beneficial for human health. In 1965, this concept was formalized by introduction of the term Probiotics by Lilly and Stilwell to define growth promoting factors produced by microorganisms. The term probiotics has been defined by the Food and Agriculture Organization and World Health Organization as Live microorganisms which when administered in adequate amounts confer a health benefit on the host'. Many types of bacteria have been used as probiotics since time immemorial. They mainly consist of lactic

acid producing bacteria belonging to two major genera namely Lactobacilli and Bifidobacteria. Certain *Bacillus* species and yeasts like *Saccharomyces boulardii* to find a place in the long list of probiotics. The mode of ingestion is either through food or in a non-food format. The probiotics generally subsist in the stomach or intestinal tract. They can potentially boost the immune system and can help in alleviation of a variety of conditions including lactose intolerance, diarrhea, colitis, hypertension, cancer, constipation, food allergies, irritable bowel syndrome and other intestinal disorders.

**Lactobacilli as Probiotics**

Lactobacilli are identified as common members of probiotics that belong to intestinal microbiota and have been considered to be an important group of bacteria in maintaining the stability of the gastrointestinal tract, in preventing intestinal infections and, generally, in supporting intestinal health (Vinderola *et al.,* 2003; Moal *et al.,* 2014; Ouwehand *et al.,* 2014). Several species of lactobacilli have the GRAS (generally regarded as safe) status and some of them have the ability to interact with intestinal epithelial cells. The mechanisms underlying probiotic effects are generally attributed to the interaction of probiotics with other microorganisms (members of the microbiota or pathogens) or with the host cells. The former type of interaction is dependent on the viability of the probiotics and is exerted by competition for nutrients or adhesion sites, direct inhibition of certain microorganisms (production of antimicrobial molecules) or increased growth of healthy components of the microbiota (Sabia *et al.,* 2014;Montiel *et al.,* 2014;Stoyancheva *et al.,* 2014). In contrast, direct interaction with the host is independent of their viability and is based on the capacity of human cells to recognize specific bacterial components or products, giving rise to responses that commonly involve the mucosa-associated lymphoid tissue and, therefore, the immune system. The ability to adhere to intestinal mucosa is reckoned as an important requirement for microorganisms intended for probiotic use, allowing at least a temporary colonization of the human and animal intestinal tract. Several factors contribute to the interaction of probiotics with the host tissues, such as cell surface hydrophobicity and auto-aggregation (Kos *et al.,* 2003), and cell surface proteins *i.e.* Sortase Dependent Proteins (SDP's), Mucin Binding Proteins (MUB), Fibronectin Binding Proteins (FnBPs) as well as Surface layer proteins(S-layer proteins).

**Structural Characterization of S-layer proteins**

The diverse structures of the cell wall associative components strongly reflect adaptations of organisms to specific ecological and environmental conditions. It has been reported that not only the live bacteria but also these active cell wall associative component play an important role in exhibiting probiotic functions like aggregation, adhesion, pathogen exclusion and immunomodulation. Among these, the S-layer proteins are well characterized cell wall associative components which are non-covalently attached to the underlying cell surface. S-layers are crystalline arrays of proteinaceous subunits located at the outermost part of the cell wall in several species of the genus *Lactobacillus*, as well as in many other bacteria and Archaea (40-200 kDa). Lactobacilli S-layers are relatively small, 25 kDa to 71 kDa in size, whereas the molecular masses of S-layers in other bacterial species range up to 200 kDa (Sara *et al.,* 2000). S-layers are normally 5–15 nm thick possessing a smoother outer surface compared with a more structured inner surface. Each S-layer forms a highly porous structure with pores of an identical size (2-8 nm) and morphology. Based on electron microscopy, the S-layer subunits are composed of lattices with oblique, square or hexagonal symmetry (Figure 1) (Sara *et al.,* 2000). The oblique lattice type was identified in the S-layers of *L. acidophilus* (Smit *et al.,* 2002), *L. brevis* (Jakava-Viljanen *et al.,* 2002) and *L. helveticus* and the hexagonal lattice type in *L. casei* and *L. buchneri*. The S-layer subunits are non-covalently linked to each other and to the supporting cell envelope, and can be disintegrated into monomers by denaturing agents such as urea or guanidine hydrochloride, by metal-chelating agents or by cation substitution. These proteins are rich in

acidic and hydrophobic amino-acids but have a low predicted pI (Sara *et al.,* 2000). However, lactobacilli proteins have high predicted pI (9.4-10.4) (Hynonen *et al.,* 2013). The lattice symmetry is of oblique or hexagonal type (Avall-Jaaskelainen *et al.,* 2005). The presence of S-layer has now been described in several species of lactobacilli such as *L. acidophilus, L. helveticus, L. brevis, L. buchneri, L. bulgaricus, L. plantarum, L. crispatus,L. kefir and L. parakefir*(Mobili *et al.,* 2010;Sun *et al.,* 2013). However, the species *L. fermentum*, *L. delbrueckii* subspecies *bulgaricus* and *L. casei* are currently considered as non-S layer producers (Hynonen *et al.,* 2013). All the lactobacilli S-layer proteins are preceded by a 25-32 amino-acid signal peptide (Avall-Jaaskelainen *et al.,* 2005).S-layer proteins can account for 10 to 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 (Goh *et al.,* 2009; Sleytr *et al.,* 1999). Functions of S-layers are not fully known. However, they have been reported to act as protective coat, determination and maintenance of cell shape, molecular sieve or ion trap or as a mediator of adhesion sites for host cells or surface recognition and virulence to pathogens (Avall-Jaaskelainen *et al.,* 2005). S-layer proteins also find application as a vehicle for the delivery of biologically active compounds, such as drug molecules, antibodies, enzymes and vaccine antigens (Sleytr *et al.,* 2007).S-layer proteins of both Gram positive and Gram negative have two structural regions; one involved in the attachment of the S-layer subunit to the cell envelope and another in S-layer assembly. Both self-assembly and cell wall binding regions have been characterized in the S-layer protein SA of *L.acidophilus* ATCC 4356 (Smit *et al.,* 2002) and CbsA of *L. crispatus* JCM 5810 (Antikainen *et al.,* 2002). It was reported that sequences of surface layer SA are homologous at C terminal mediating attachment to cell wall and the N-terminal being variable is responsible for self-assembly.

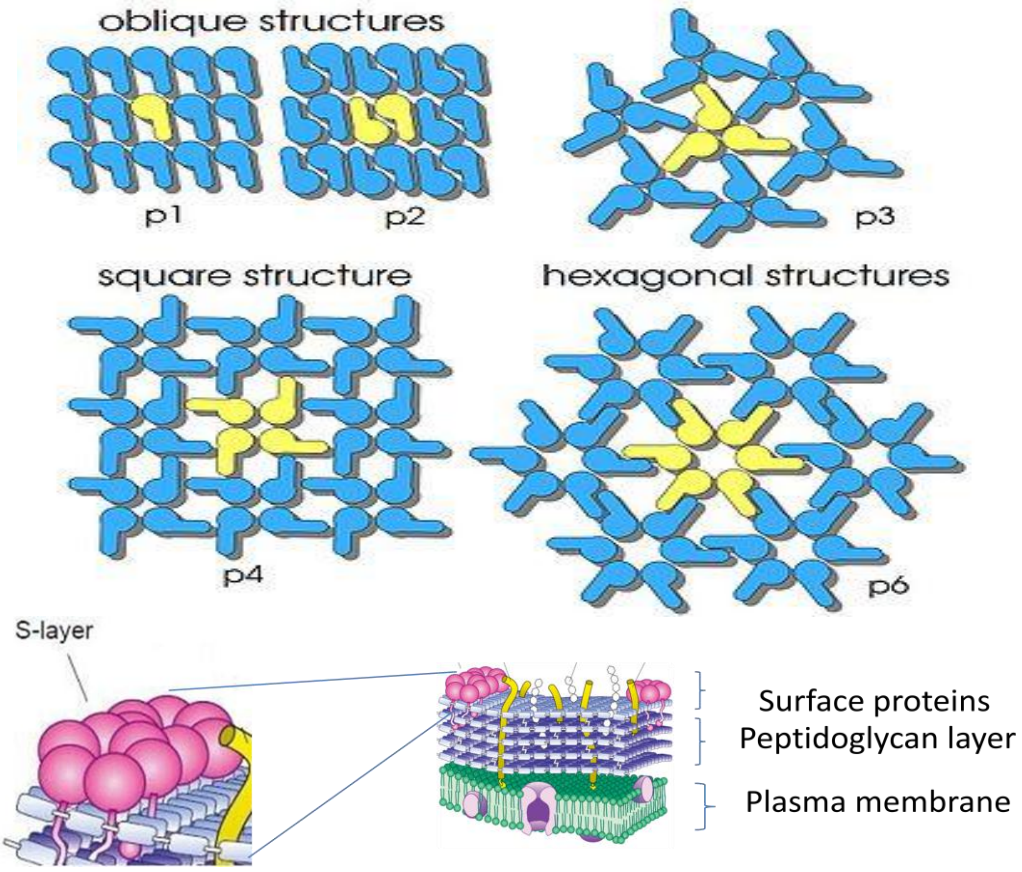


**Figure 1. Diagrammatic representation of different symmetries of S-layer protein**

S-layer like proteins have also been described in genera lactobacilli by Ventura*et al.*(2002) who identified and sequenced the genes encoding the aggregation-promoting factor (APF) protein fromsix different strains of *L. johnsonii* and *L. gasseri* which shared several characteristics *i.e.* amino acid composition, physical properties, and genetic organization. This was found to be similar to Surface layer proteins and suggested that APF protein is a novel surface protein of the *L. acidophilus* B-homology group which might belong to anS-layer-like family.

**S-layer protein in *Lactobacillus* spp.**

Cell surface of several strains of lactobacilli are covered by S-layer protein providing protective barrier against hostile gut environment as well as adhesive properties for colonization in the GI tract to express their beneficial functions (Johnson *et al.,* 2016; Wuyts *et al.,* 2017). In a recent study carried out by Xue*et al.*(2013) it was reported that removal of S-layer proteins from the *Lactobacillus* (treated with 5 M LiCl) reduced inhibition of enteric pathogens as revealed in exclusion, competition and displacement assays suggesting that S-layer proteins were involved in the adhesion of probiotics.

**S-layer protein of *Lactobacillus acidophilus***

S-layer protein of 43 kDa from *L. acidophilus* ATCC 4356 was purified by Boot *et al.* (1993) by the treatment of 4.0 M guanidine hydrochloride followed by cation-exchange chromatography. S-layer protein of *L. acidophilus* ATCC 4356 was found to be positively charged and had a predicted isoelectric point of 9.40. Later, Frece *et al.* (2005) purified S-layer protein of *L. acidophilus* M92 which had a molecular weight 45 kDa. The presence of *slp*A gene was confirmed by southern blotting using *L. acidophilus* ATCC 4356 as positive control since it possesses *slp*A gene. They further investigated the protective role of S-layer protein of *L. acidophilus* M92 and *L. acidophilus* ATCC 4356 by exposing them to gastric and pancreatic juice before and after treatment with LiCl. It was reported that survivability of *L. acidophilus* M92 and *L. acidophilus* ATCC 4356 in gastric and pancreatic juice was reduced after treatment with LiCl in comparison to untreated cells that confirmed the protective role of S-layer protein in gastrointestinal tract. S-layer protein was found in all growth phases of *L. acidophilus* M 92 at 37, 45 and 50°C. *L. acidophilus* M92 was able to grow at 50°C but it did not grow at this temperature after removal of S-layer. S-layer protein from *L. acidophilus* M92 had the ability of aggregation or adhesion to the porcine ileal epithelial cells. After removal of S-layer protein with the treatment of 5M LiCl, it was observed that it reduced the adhesiveness of *L. acidophilus* M92 to epithelial cells, thus establishing that S-layer protein promoted the adhesiveness of the strain *L. acidophilus*M92 (Frece *et al.,* 2005). Buck*et al.*(2005) also reported cell surface proteins in *L. acidophilus* thatmay promote attachment to intestinal tissues. They identified four proteins potentially involved in adhesion using the genomic data and constructed isogenic mutations in genes encoding a mucin-binding protein, a fibronectin-binding protein, a surface layer protein, and two streptococcal R28 homologs using homologous recombination. A significant decrease in adhesion to Caco 2 cells was observed in the fibronectin-binding protein mutant (76%), mucin-binding protein mutant (65%) and surface layer protein mutant (84%). This study demonstrated that multiple cell surface proteins in *L. acidophilus* NCFM can individually contribute to the organism's ability to attach to intestinal cells *in vitro*. Konstantinov *et al.* (2008) on the other hand, examined the interaction of *L. acidophilus* NCFM and its cell wall component S-layer protein to modulate the dendritic cells and T-cell function. Dendritic cells are antigen presenting cells and expressed DC-specific ICAM-3-grabbing non-integrin (DC-SIGN) that is a C-type lectins receptor (CLRs). S-layer protein, SlpA of *L. acidophilus* NCFM is the first probiotic protein that acts as a DC-specific ICAM-3-grabbing non-integrin ligand. This protein interacted with receptor DC-SIGN resulting in production of anti-inflammatory cytokines IL-10, IL-6 in higher amount and stimulation of both humoral and adaptive immune responses. A knockout

mutant of *L. acidophilus* NCFM lacking the surface (S) layer Aprotein (SlpA) had significantly reduced binding to DC-SIGN.

An interesting study carried out by Acosta *et al.* (2008) pointed towards the murein hydrolase activity of S-layer protein of *L. acidophilus* ATCC 4356 against *Salmonella enterica* serovar Newport indicating that murein hydrolase activity of S-layer protein provided additional potential to surviving of probiotic *Lactobacillus* in mixed micro-flora of gastrointestinal tract. By comparing a purified SlpA subunit from *Lactobacillus acidophillus* and a mutant expressing the major SlpA, SlpA was shown to be a probiotic factor able to bind to the C-type lectin that is a host immune receptor SIGNR3. This modulated regulatory signals, which resulted in mitigation of colitis, maintenance of healthy gastrointestinal micro-biota, and protection of gut mucosal barrier function in mice (Lightfoot *et al.,* 2018). In addition, S-layer protein (SLP and SLAPs) from *Lactobacillus acidophilus* NCFM, have been implicated in both mucosal immunomodulation and adhesion to the host intestinal epithelium (Johnson *et al.,* 2017). Recently, Li *et al.* (2011b) reported that S-layer protein from *L. acidophilus* mediated inhibition of Salmonella-induced apoptosis in Caco-2 human colon epithelial cells. Normal apoptosis process was found in response to bacterial infection which may function to delete infected and damaged epithelial cells but in some cases pathogenic bacteria hijack the host apoptotic pathway to facilitate its pathogenesis. *Salmonella* has the ability to delay the apoptosis for 12–18 h after infection in human intestinal epithelial cell lines. The delay in onset of epithelial cell apoptosis may be critical for some intracellular pathogens, since it provides sufficient time for proliferation and adaptation to the intracellular environment to cause a large amount of cell injury. Apoptosis acted via the activation of caspase-3 pathway that is a key executioner caspase in the proteolytic cascade that leads to apoptotic cell death, and cleaves a number of structural proteins during the execution phase of apoptosis. The phosphorylation of extracellular regulated kinesis such as ERK-1and ERK-2 was involved in activation of caspase-3 pathway and S-layer proteins were able to suppress Salmonella-induced ERK1/2 phosphorylation. S-layer proteins have the ability to autoassemble on bacterial surfaces and hence might associate with the *Salmonella* surface, and could interact with specific sites on the bacterial surface involved in the first step of mucosal infection. In a similar study by the same authors Li *et al.* (2011b) it was reported that S-layer proteins could antagonize *S.typhimurium* infection by protecting against F-actin cytoskeleton rearrangement and activation of mitogen -activated protein kinase (MAPK) signaling pathway. F-actin is fine, long, and uniform distribution of thick fibers like structure these are located at the center of the cells. This F-actin cytoskeleton is essential for the formation and maintenance of intestinal cell barrier integrity. When caco-2 cells were treated with *S. typhimurium* alone, it was observed that a rapid decrease in trans-epithelial electrical resistance (TER), and production of several pro-inflammatory mediators including the chemokine interleukin 8 (IL-8), F-actin disruption and induction of phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun amino-terminal kinase (JNK) and p38 occurred. On the other hand, when *S. typhimurium* was co-incubated with S-layer proteins, it induced Caco-2 cell F-actin rearrangement was reduced, and the *S. typhimurium* induced TER decrease and interleukin 8 (IL-8) secretions were attenuated. Additionally, *L. acidophilus* S-layer proteins could inhibit *S. typhimurium* induced phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun amino-terminal kinase (JNK) and p38.

In a recent study, Martinez *et al.* (2012) reported that S-layer protein from *L. acidophilus* had the ability to inhibit the junin virus infection through pathogen exclusion. Junin virus belongs to Arenaviridae family. It causes Argentine hemorrhagic fever leading to major alteration within the neurological and immune system. Dendritic cells expressing DC-specific ICAM-3-grabbing non-integrin protein (DC-SIGN), which is a cell surface receptor and recognized through many molecules such as mannose and fructose glycans that are present

on microbial and viral surfaces, interact with *L. acidophilus.* Complete inhibition of junin virus infection was shown when they were treated with purified S-layer protein from *L. acidophilus* ATCC 4365. This effect was not observed in cell lines that do not express these DC-SIGN and post infection treatment with S-layer, because S-layer protein was not directly interacting with viral particles but the target of this protein was DC-SIGN. This inhibitory effect is a novel characteristic of the S-layer protein of *L. acidophilus*, which could account for the pathogen exclusion effect described for this probiotic bacterium. S-layer protein from *L. acidophilus* ATCC4356 is stable in harsh conditions of gastrointestinal system such as simulated gastric fluid (SGF) with pH 2 up to 5 min. with and without pepsin and it is also stable in all the simulated intestinal fluids. It opens interesting perspectives in the using and development of this S-layer as a protective coat for oral administration of unstable drug nanocarriers (Eslamia *et al.,* 2013).

**S-layer protein of*Lactobacillus crispatus***

Two different S-layer-expressing strains *L. crispatus* JCM 5810 and *L. acidophilus* JCM 1132 were investigated by Toba *et al.* (1995) for their adherence to proteins of the mammalian extracellular matrix (ECM). Functional heterogeneity among the S layer proteins was observed in lactobacilli. *L. crispatus* JCM 5810 was found to adhere efficiently to immobilized type IV and I collagens and laminin but showed lower binding efficiency with type V collagen and fibronectin. On the other hand *L. acidophillus* JCM 1132 did not exhibit detectable adhesiveness. On incubation of purified preparations of S-layer proteins with radiolabelled mammalian extracellular matrix proteins, S-layer of *L. crispatus* JCM 5810 efficiently bound $^{125}$I-labelled type IV collagen and poor binding was *L. acidophilus* JCM 1132. Binding of $^{125}$I-collagen IV to the JCM 5810 S-layer protein was effectively inhibited by unlabelled type I and IV collagens but not by type V collagen, laminin, or fibronectin. It was concluded that *L. crispatus* JCM 5810 has the capacity to adhere to human subintestinal extracellular matrix via a collagen-binding S-layer. Later, working on the same strain *L. crispatus* JCM 5810, Horie *et al.* (2002) reported that the S-layer protein (CbsA) inhibited the adhesion of three strains of diarrhea causing *Escherichia coli* to the matrigel. CbsAof *L. crispatus* JCM 5810 inhibited pathogenic *E. coli* from adhering to basement membrane via competition with laminin molecules for binding sites.

Chen *et al.* (2009) studied the *in vitro* probiotic properties exerted by the S-layer proteins of *L. crispatus* ZJ001 isolated from pig intestine and compared them with *L. acidophilus* ATCC 4356. The strain ZJ001 exhibited tolerance to acid and bile salts, high adhesive ability due to having autoaggregation property and competition against pathogenic organisms as compared with ATCC4356. It was shown that removal of the S-layer proteins with the treatment of 5M LiCl reduced their probiotic activity such as tolerance against acid and bile salt and adhesion to the HeLa cells. The functional role of the S-layer proteins in adhesion was also confirmed by the antibody mediated inhibition assay using the polyclonal antibody against the S-layer protein. The S-layer protein from *L. crispatus* ZJ001was found to prevent the adhesion of potential pathogen like *E. coli* 0175:H7 upon co-incubation of *E. coli* O157:H7 or *S. typhimurium* to HeLa cells through a competitive exclusion mechanism. These results suggest that *L. crispatus* ZJ001 possessed probiotic properties and the S-layer proteins are involved in the adhesion and competitive exclusion of pathogens *E. coli* O157:H7 or *S. typhimurium* to HeLa cells. Recently, Hu *et al.* (2011) reported two functional regions in S-layer protein *i.e.* self-assembly domain and the cell wall-targeting domain. These regions have been characterized in the S-layer proteins SA of *L. acidophilus* ATCC 4356, CbsA of *L. crispatus* JCM 5810, and SlpA of *L. brevis* ATCC 8287. Sequences of S-layer proteins SA from *L. acidophilus* ATCC 4356 and CbsA from *L. crispatus* JCM 5810 showed similarity in C-terminal region, responsible for anchoring the S-layer subunits to the bacterial cell wall, and the more variable N-terminal regions were involved in the self-assembly process. On the

other hand, in case of SlpA of *L. brevis* ATCC 8287, the domains responsible for the self-assembly process (C-terminal region) and cell wall binding (N-terminal region) were located in reverse order compared to SA and CbsA. It was reported that S-layer protein exists in the form of lattice that covered the whole cell surface of bacteria. These properties make S-layer proteins an attractive anchor for the development of microbial cell-surface display systems. To evaluate the possibility of the LcsB region (C-terminal region) of SlpB from *L. crispatus* K2-4-3 as a cell wall anchor, the LcsB region was tagged with green fluorescent protein (GFP) and beta-galactosidase gene and expressed in *E. coli* host. Purified GFP-LcsB and Gal-LcsB was added to various LAB cells *in vitro,* and the binding was viewed by using whole-cell fluorescence measurement and enzymatic activity assays, indicating that both proteins retained their function while presented on the cell surface. Three different S-layer protein genes (*slp*A, *slp*B, and *slp*C) were identified in *L. crispatus* K313, sequenced and characterized in detail by Sun*et al.,*(2013). Although, *slp*A was silent but *slp*B gene was predominantly expressed in *L. crispatus* K313, and was further investigated for its functional domains by producing a set of N- and C-terminally truncated recombinant SlpB proteins in *Escherichia coli.* It was reported that S-layer proteins of *L. crispatus* K313 possessed two functional domains *i.e.* C-terminus and N-terminus domain. C-terminus domain of SlpB was responsible for the anchoring of the S-layer to the cell wall and N-terminal domain of SlpB could confer adhesiveness to types I and IV collagen.

**S-layer protein of *Lactobacillus helveticus***

S-layer protein from *L. helveticus* ATCC 12046 was extracted by Lortal*et al.*(1991) through the treatment of 5M LiCl and they reported that N-terminal region of these proteins are rich in alanine, threonine, asparagine and aspartic acid and shown the reformed ability on the surface of bacterial cell after removal with LiCl. On the other hand, Ventura *et al.*(2000) used S-layer protein gene as a molecular marker for identification of *L. helveticus*. Heterogeneity of putative surface layer proteins in *L. helveticus* was also reported by Gatti *et al.* (2005) who characterized S-layer-encoding genes from 21 *L. helveticus* strains collected from different sources. Phylogenetic analysis based on the identified S-layer genes revealed two main clusters, one which includes a sequence similar to that of the *slp*H1gene of *L. helveticus* CNRZ 892 and a second cluster which includes genes similar to that of *prtY*. The data showed that amino acid sequence was highly conserved in N-terminal and C-terminal regions and highly variable in the central parts of the protein. The functional property of surface-layer protein (Slps) extracts from *L. helveticus* R0052 was investigated by Johnson-Henry *et al.* (2007). It was reported that S-layer protein inhibited enterohaemorrhagic *E. coli* O157:H7 adhesion to epithelial cells through the competition of binding site on intestinal epithelial cell. In a similar study, Beganovic *et al.* (2011) characterized the functionality of S-layer protein from the probiotic strain *L. helveticus* M92. Native S-layer protein (SlpA) was isolated from *L. helveticus* M92 by the 5M LiCl, identified by SDS-PAGE and liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. The *slp*A gene sequence from *L. helveticus* M92 showed the high level of sequence homology to the other *Lactobacillus* S-layer genes. They further reported the auto-aggregation and co-aggregation ability of S-layer protein with *L. helveticus* M92 cells and *L. helveticus* M92 with *S. typhimurium* FP1. However, these processes were negatively affected after removal of S-layer protein from the cell surface of *L. helveticus* M92. Immunomodulatory effect was also seen through *in-vitro* study on immunizing mice with purified *L. helveticus* M92 SlpA protein and with *L. helveticus* M92 cells without SlpA. After the oral immuniztion of mice with purified SlpA, the levels of serum IgA, IgG, and IgM antibodies were significantly higher in comparison to the levels of these antibodies in the group of mice immunized with *L. helveticus* M92 cells without SlpA.Isolated Single-chain variable-fragment antibodies (scFvs) is promising for the development of a rapid and accurate ELISA-based detection assay for the *L. helveticus* MIMLh5 S-layer protein to characterize the

potential immunomodulatory properties of dairy based foods (Stuknyte *et al.,* 2014). S-layer protein from *L. helveticus* MIMLh5 showed anti-inflammatory effects by reducing the activation of NF-κB on the intestinal epithelial Caco-2 cell line and modulate the immune system by triggering the expression of proinflammatory factors, tumor necrosis factor alpha and COX-2 in the human macrophage cell line U937 via recognition through Toll-like receptor 2 (Taverniti *et al.,* 2013).S-layer proteins from six strains of *L. helveticus* were identified, sequenced and characterized. Strong amino acid sequence conservation of all Slp studied and PCR analysis revealed that five of the examined strains did not have *slpH* genes. So, *SlpH* genes cannot be used as molecular markers for *L. helveticus* (Wasko *et al.,* 2014).

**S-layer protein of*Lactobacillus brevis***

Avall-Jaaskelainen *et al.* (2008) characterized domains responsible for self-assembly and cell wall binding of the S-layer protein of *L. brevis* ATCC 8287. They reported SlpA as a two-domain protein in which the order of the functional domains is reversed compared to other characterized *Lactobacillus* S-layer proteins, and emphasizes the diversity of potential cell wall receptors despite similar carbohydrate binding sequence motifs in *Lactobacillus* S-layer proteins.In order to understand the role of S-layer protein in alleviation of calf diarrohea, Khang *et al.,* (2009) constructed a chimeric gene encoding enhanced green fluorescentprotein (EGFP) and a S-layer protein from *L.brevis* KCTC3102, and/or two copies of the Fc-bindingZ-domain, a synthetic analogue of the B-domain of proteinA and expressed in *E. coli* BL21(DE3). They further reported that feeding a mixture of recombinant S-layer fusion proteins and antibodies against neonatal calf diarrhoea (coronavirus, rotavirus*, E. coli* and *S. typhimurium*) to calves resulted in 100% prevention of neonatal calf diarrhoea syndrome($p<0.01$), whereas feeding antibodies only resulted in 56% prevention. Angle X-ray scattering (SAXS) and zeta potential measurements are useful techniques for characterizing the reassembly properties of crystalline structure formed by S-layer protein SlpA of *L. brevis* ATCC 8287 on positively charged liposomes (Kontroa *et al.,* 2014).

**Genetics of S-layer proteins**

Most species of the lactobacilli possess S-layer in the form of outermost coat of the cell. S-layer protein encoding genes have been cloned and sequenced from two *L. brevis* strains (Vidgren *et al.,* 1992;Jakava-Viljanen *et al.,* 2002), two *L. acidophilus* strains (Boot *et al.,*1993;Buck *et al.,* 2005), one *L. helveticus* strain (Callegari *et al.,* 1998), one *L. crispatus* strain (Sillanpaa *et al.,* 2000) and seven *L. gallinarum* strains (Hagen *et al.,* 2005). The S-layer protein genes are highly expressed. These S-layer proteins are encoded by more than one gene that is present in the genome of the *Lactobacillus*, but not all of them are necessarily expressed at the same time. S-layer protein genes are preceded by more than one promoter, which may not only increase the transcription efficiency but also offers a way to regulate the S-layer gene expression in response to, for instance, growth stage or environmental conditions. S-layer protein encoding genes have been cloned and sequenced from different species of *Lactobacillus* such as *L. brevis*, *L. acidophilus*, *L. helveticus*, and *L. crispatus*. Additionally, *L. amylovorus, L. buchneri, L. gallinarum, L. kefir* and *L. parakefir* have also been shown to possess an S-layer, but their S-layer protein genes have not yet been sequenced. The presence of more than one S-layer protein genes seems to be quite common for lactobacilli, for example, two S-layer protein genes '*cbs*A' and '*cbs*B' were identified in *L. crispatus* JCM 5810 (Sillanpaa *et al.,* 2000). Three of the S-layer protein genes '*slp*B', '*slp*C', and '*slp*D' were isolated, sequenced, and studied for their expression in *Lactobacillus brevis* ATCC 14869 under different environmental conditions and it was found that '*slp*B' and '*slp*D' expressed in aerobic conditions and '*slp*B' expressed when cells were grown in anaerobic condition. On the other hand, '*slp*C' was a silent gene under the growth conditions (Jakava-Viljanen *et al.,* 2002), and two out of the three S-layer protein genes, *i.e.*'*slp*B' and '*slp*C', were found to be located adjacent to each other in parallel orientation whereas the third gene, '*slp*D', was

not closely linked to the *slp*B-*slp*C gene region. On the other hand, in *L. acidophilus* ATCC 4356, the two S-layer protein genes, '*slp*A' and '*slp*B', were in opposite orientation to each other. These studies indicate that the genetic arrangement of the multiple S-layer protein genes in lactobacilli is strain-dependent, and no general consensus structure of their genetic organization can be deduced. After DNA-DNA hybridizations, S-layer protein gene '*slp*A' from *L. brevis* ATCC and chromosomal DNA of *L. buchneri* DSM 20057 indicates that *L. buchneri* DSM 20057 contains homologous sequences to slpA. In a recent review by Hynonen *et al.* (2013) it has been reported that confirmation of the presence of S-layer till today relies on electron microscopy since there is low overall similarity among S-layer protein genes and lack of universal signature sequence.

**Production of S-layer proteinby recombination DNA technology**

Native S-layer proteins have already demonstrated their great potential in adhesion, pathogen exclusion and immunomodulation. Genetic approaches have further opened up the possibility of heterologous production and cloning and expression of S-layer fusion proteins. While, the heterologous expression of S-layer proteins or its receptor-binding region can change non-adhesive lactic acid bacteria into adhesive, cloning and expression of '*slp'* gene in hosts such as *E. coli* can help in the large scale production of recombinant S-layer protein. The production of recombinant S-layer protein is a very crucial and a prospective step for future research applications of S-layer in understanding their precise role in probiotic functionality specifically with regard to their efficacy in alleviation of gastrointestinal disorders. In this context, as early as in 1993, Boot *et al.* (1993) expressed the S-layer protein of *L. acidophilus* ATCC 4356 in *E. coli* and sequenced the corresponding gene.On screening the genomic library of *L. acidophilus* ATCC 4356 prepared in lambda-EMBL3, the *slp*A gene, coding for the surface layer protein, was found to be located entirely on the 4.0-kb fragment. The nucleotide sequence encoded a protein of 444 amino-acids, the first 24 resembling a putative secretion signal. The predicted isoelectric point of 9.4 was in agreement with the data obtained during purification. However, the S-layer protein expressed from recombinant *E. coli* had slightly higher molecular weight than the native purified protein. The expression of the entire S-protein or of large, C-terminally truncated S-proteins was unstable in *E. coli*.Working on similar lines, Callegari *et al.* (1998) cloned and sequenced S-layer protein gene from *L. helveticus* CNRZ 892 in *E. coli* and expressed in heterologous host *Lactococcus lactis* MG1363. The molecular weight of S-layer protein from *L. helveticus* CNRZ 892 was found to be 43 kDa. The ORF of '*slp*H' contained 1320 nucleotides and is preceded by a putative ribosome-binding site (AGGAGG) located 9 nucleotide upstream of the 'AUG' start codon. It is followed by a single stop codon (UAA) and a possible ρ-independent transcription terminator. Gene '*slp*H' contains 12 direct repeated sequences ranging from 7 to 16 nucleotides. The '*slp*H' was expressed in *L. lactis* MG1363 using lactococcal expression vector pMG36e having lactococcal promoter P32. The expressed S-layer protein showed the same molecular mass as the *L. helveticus* CNRZ 892 '*slp*H', as judged by SDS-PAGE. Martinez *et al.* (2000) expressed '*cbs*A' gene that encoded the collagen-binding S-layer protein of *L. crispatus* JCM5810 into *L. casei* ATCC 393 which lacks S-layer and was not able to bind to collagen or colonize the GI tract. The S-layer protein was not retained on the surface of the host bacteria when expressed alone but after fusion of CbsA to the cell wall sorting signal of the proteinase, PrtP, of *L. casei*, CbsA was presented at the surface of host bacterium. Antiserum was raised against CbsA protein to confirm the surface location of CbsA in *L. casei.* The binding capacity of expressed protein CbsA to collagen types I and IV was also evaluated. The result of this study showed that the collagen-binding capacity of an S-layer protein can be transferred to another lactic acid bacteria host. Acosta *et al.* (2008) reported that S-layer protein of *L. acidophilus* ATCC 4356 had murein hydrolase activity against cell wall of *Salmonella enterica* serovar Newport. Endopeptidase activity of Slp was confirmed by

zymogram and Western blotting with an antibody against the S-layer. On the basis of amino acid sequence comparisons, the hydrolase activity was found to be located at the C terminus. Carboxy-terminal domain of the S-layer protein was amplified and 968-bp amplicon was cloned in pHCMC05 shuttle plasmid vector. The recombinant plasmid was found to be highly unstable and showed decreased growth. The authors ascribed this failure due to the peptidoglycan structure of this host bacterium, since it was a substrate for the lytic activity of this S-layer protein having hydrolase activity. This recombinant vector was introduced into *Bacillus subtilis* 168 and the heterologus product was confirmed by western blotting resulting in the functional verification of the enzymatic activity. The murein hydrolase activity of S-layer protein provides additional potential to survival of probiotic lactobacilli in mixed micro-flora of gastrointestinal tract.

In order to understand the role of S-layer protein in alleviation of calf diarrohea, Khang*et al.*(2009) constructed a chimeric gene encoding enhanced green fluorescent protein (EGFP) and a S-layer protein from *L.brevis* KCTC3102, and/or two copies of the Fc-bindingZ-domain, a synthetic analogue of the B-domain of proteinA and expressed in *E. coli* BL21(DE3). They further reported that feeding a mixture of recombinant S-layer fusion proteins and antibodies against neonatal calf diarrhoea (coronavirus, rotavirus*, E. coli* and *S. typhimurium*) to Hanwoo calves resultedin 100% prevention of neonatal calf diarrhoea syndrome ($p<0.01$), whereas feeding antibodies only resulted in 56%prevention.Chen *et al.,*(2009) sequenced and expressed S-layer genes (*slp*A and *slp*B) of *L. crispatus* (*L. crispatus*) ZJ001 in *E. coli* in order to characterize their functions. However, only *slp*A gene could be expressed successfully. Both the genes were highly homologous at C-terminal to other *Lactobacillus* S-layer proteins, but were substantially variable at both N-terminal and middle regions. The mature SlpA exhibited 47% amino acid sequence identity to SlpB. Both the recombinant proteins were able to bind HeLa cells to the extent similar to the native S-layer. The cell binding domains were in N-terminal regions as revealed by high binding of truncated peptides SlpA2-228 and SlpB2-249. The authors further reported that *slp*A gene could be targeted to display foreign proteins on the bacterial surface of ZJ001 as a potential mucosal vaccine vector.

**Biological Significance of *Lactobacillus* S-layer proteins**

Lactobacilli S-layer proteins mainly exhibit adhesion to epithelial cells and to intestinal components like mucus or ECM proteins besides providing as a protective coat against harsh environmental conditions and inhibition of pathogens besides immunomodulation.

**Aggregation properties of S-layer protein**

Aggregation is an interaction process between microorganisms. The interacting organisms may be genetically equal or different and hence, the term 'autoaggregation' and 'coaggregation' respectively. The aggregative capacity of bacteria could be considered as a potentially probiotic attribute since, auto-aggregation appears to be the first step for adhesion to intestinal epithelial cells and bacteria with co-aggregation abilities may form a barrier preventing colonization by pathogenic microorganisms. The aggregation property of *Lactobacillus* has been attributed to S-layer proteins. Auto-aggregation of *L. helveticus* M92 cells and co-aggregation of *L. helveticus* M92 with *S. typhimurium* FP1 has been reported and it has been observed that removal of the S-layer by treatment with 5M LiCl reduces the auto-aggregation and co-aggregation ability of the strains *L. acidophilus* M92.*L. crispatus* ZJ001 is also reported to lose its auto-co-aggregation ability after removal of S-layer protein from the cell surface with the treatment of LiCl (Chen *et al.,* 2007). The removal of the S-layer by treatment with 5M LiCl also reduced the auto-aggregation of *L. kefir* CIDCA 8321 (Garrote *et al.,* 2004) and its co-aggregation with the yeast S*accharomyces lipolytica* CIDCA 812 (Golowczyc *et al.,* 2007), strongly suggesting that these processes are mediated by lactobacillar S-layer proteins. Similar findings were observed by Mobili *et al.,* (2010) who suggested a correlation between the

structure of S-layer glycoproteins and the aggregation property of whole bacterial cells. In a recent study, Carasi *et al.* (2012) reported that the S-layer proteins from aggregating *L. kefir* strains showed a higher inhibitory ability to antagonize the effect of Clostridial toxins.

**Adhesion ability with epithelial cells and ECM proteins**

The S-layers of certain lactobacilli have demonstrated to act as adhesins, mediating the binding of these bacteria to specific components of the extracellular matrix (ECM). Direct experimental evidence of this activity has been obtained for the S-layer proteins of *L. crispatus* JCM 5810 (CbsA), *L. brevis* ATCC 8287 (SlpA) and *L. brevis* OLL2772. The adhesion to epithelial cells has also been attributed to the S-layers. Toba *et al.* (1995) reported functional heterogeneity among the S-layer proteins in two different S-layer-expressing strains *L. crispatus* JCM 5810 and *L. acidophilus* JCM 1132 for their adherence to proteins of the mammalian extracellular matrix (ECM). *L. crispatus* JCM 5810 was found to adhere efficiently to immobilized type IV and I collagens and laminin but showed lower binding efficiency with type V collagen and fibronectin. Chemical removal of the S-layer proteins significantly reduced the adhesion of *L. brevis* ATCC 8287 to the human intestinal cell lines Caco-2 and Intestine 407, the endothelial cell line EA-hy926 and the urinary bladder cell line T24 (Hynonen *et al.,* 2002), the adhesion of *L. acidophilus* ATCC 4356 and M92 to avian and porcine intestinal epithelial cells *in vitro* (Sillanpaa *et al.,* 2000; Frece *et al.,* 2005) and the adhesion of *L. crispatus* ZJ001 to HeLa cells (Chen *et al.,* 2009) indicating the involvement of S-layer in lactobacilli adhesion to different cell lines.

**Role in Pathogen Exclusion**

S-layer proteins with adhesive properties could contribute to lactobacilli probiotic activity by the inhibition of the binding of pathogens to host tissues. This can be achieved through direct competition for attachment sites on human intestinal cells, ECM and mucus proteins, or by the blockage of pathogen surface adhesins. Several studies have reported that S-layer proteins from lactobacilli inhibited the adherence of pathogens such as *E. coli*, *E. coli* O157:H7 or *Salmonella typhimurium* to intestinal cells (Horie *et al.,* 2002; Chen *et al.,* 2009). Johnson-Henry *et al.* (2007) also reported that surface-layer protein (Slps) extracts from *L. helveticus* R0052 inhibited enterohaemorrhagic *E. coli* O157:H7 adhesion to epithelial cells through the competition of binding sites on intestinal epithelial cell. S-layer protein from *L. acidophilus* was also reported to mediate inhibition of Salmonella-induced apoptosis in Caco-2 human colon epithelial cells (Li *et al.,* 2011a;b) recently; Martinez *et al.*(2012) reported that S-layer protein from *L. acidophilus* had the ability to inhibit the junin virus infection through pathogen exclusion. In a similar study, Xue*et al.*(2013) reported that removal of S-layer proteins from the *Lactobacillus* strains (treated with 5 M LiCl) reduced *Clostridium difficile*, *Shigella* and *Salmonella* inhibition activity as revealed in exclusion, competition and displacement assays, thereby, suggesting that S-layer proteins were involved in the adhesion of probiotics. Xue *et al.* (2014) also studied the role of S- layer proteins in increased adhesion and competitive exclusion of pathogens. S-layer protein from *Lactobacillus acidophilus* inhibit bacterial infection by binds to the cellular receptor Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin; CD209 (Acosta *et al.,* 2016).

**Immunomodulatory effects**

S-layer protein of *L. acidophilus* NCFM has ability to modulate the dendritic and T-cell function. S-layer protein, slpA of *L. acidophilus* NCFM is the first probiotic protein to act as a DC-specific ICAM-3-grabbing non-integrin ligand. This protein interacts with receptor DC-SIGN resulting in production of anti-inflammatory cytokines IL-10, IL-6 in higher amount and stimulation of both humoral and adaptive immune responses (Konstantinov *et al.,* 2008; Carasi *et al.,* 2017). In a later study, Beganovic *et al.* (2011) investigated the immunomodulatory effect in an *in-vitro* study by immunizing mice with purified *L. helveticus* M92 SlpA protein and with *L. helveticus* M92 cells without SlpA. After the oral immunisation

of mice with purified SlpA, the levels of serum IgA, IgG, and IgM antibodies were significantly higher in comparison to the levels of these antibodies in the group of mice immunized with *L. helveticus* M92 cells without SlpA.S-layer protein from *L.helveticus* MIMLh5 also play an important role in stimulation of innate immune system in human (Taverniti *et al.,* 2013). Recently, it has been found that S-layer protein from *Lactobacillus kefiri* CIDCA 8348 enhances macrophages response to LPS in a $Ca^{+2}$-dependent manner (Malamud *et al*., 2017).

**Applications of S-layer proteins**

S-layer proteins find a broad spectrum of applications in biotechnology, nanotechnology, nanobiotechnology and biomimetics due to theirhigh degree of structural regularity and their self-assembly properties in several matrices as well as the substantial body of information available on their structure, genetics, chemistry, assembly and physicochemical properties.S-layer can be used as matrices for immobilization of foreign molecules such as antibodies (Breitwieser *et al.,* 1996; Lebeer*et al.,* 2017), allergens, oligosaccharide haptens or biochemically and biomedically interesting proteins. A fusion protein was constructed by gene sequence encoding the major birch pollen allergen (Betv1) with the gene encoding the bacterial cell surface (S-layer) protein.

The recombinant protein rSbsC-Betv1 contained all relevant Betv1-specific B and T cell epitopes. IFN-gamma along with IL-10 was induced by rSbsC-Betv1 but not Th2-like response, as observed after stimulation with Betv1. Moreover, rSbsC-Betv1 induced IFN-gamma synthesis in Betv1-specific Th2 cell clones, and importantly, increased IL-10 production in these cells. The fusion of an allergen to S-layer proteins reduced allergenicity with immuno-modulatory capacity in *in-vitro* conditions. The described strategy is generally applied to design vaccines for specific immunotherapy of type I allergy with improved efficacy and safety (Moll *et al.,* 2002; Vollenkle *et al.,* 2004).S-layer proteins have also been utilized as matrices for the development of dipstick-style immunoassays. A dipstick assay has been developed for prion diagnosis based on a sandwich ELISA specific for prion protein. Crystalline bacterial cell surface layers (S-layers) are used as an immobilization matrix in this assay that help in the diagnosis of human diseases (Volkel *et al.,* 2003). S-layer can be used as isoporous ultrafiltration membranes and as molecular sieves because 70% area of S-layer is covered by porous structure. The pore size of S-layer was reported from 2-8 or 3-4.5 nm. Due to having nano-meter pore size it can be used as permeable membrane (Sara *et al.,* 1992; Sleytr *et al.,* 2014). The promoters and/or the signal sequence of the *Lactobacillus* S-layer protein genes have been used for intra- or extracellular heterologous protein production in lactic acid bacteria. Non-adhesive lactic acid bacteria can be turned into adhesive by heterologous surface expression of the S-layer protein or its receptor-binding region (Martınez *et al.,* 2000; Johnson *et al.,* 2016).

The role of S-layer proteins for development of mucosal vaccines has caught the attention of several researchers due to their GRAS status. The potential of S-layer proteins as antigen carriers has also been realized since polysaccharides or proteins could be chemically linked to S-layers to elicit the immune response. Due to their intrinsic adjuvanticity, as well as their capability to surface-display proteins and epitopes (Bingle *et al.,* 1997; Umelo-Njaka *et al.,* 2001) they have potential to be used as antigen carriers. S-layer proteins can also be used as templates for the formation of regularly arranged metallic nanoparticles (Mertig *et al.,* 2001; Wuyts *et al.,* 2017). The only nano-technological application with a *Lactobacillus* S-layer to date is the study by Sampathkumar and Gilchrist, where the S-layer protein of *L. brevis* was utilized in the formation of bio-conjugates. With amine-based coupling chemistry, small molecule probes and polymers were conjugated to the *L. brevis* S-layer and the self-assembly of these bio-conjugates was studied by amine-terminated polystyrene beads as the assembly substrate. According to different microscopy analyses, these bio-conjugate coated microspheres showed a homogenous distribution of the polymer conjugates. Moreover, it was also

demonstrated using this method, that surfaces containing homogenous display of mixed monolayers can be achieved (Sampathkumar *et al.,* 2004). Due to having stability at harsh conditions of gastrointestinal system it has a potential application as protective coat for oral administration of unstable drug nano-carriers (Eslamia *et al.,* 2013).

## CONCLUSION

In conclusion, it is clear that S-layer is the outer boundary of *Lactobacillus*. S-layers have been shown to function in pathogen exclusion, immunomodulation and as adhesins, mediating binding of bacteria to the host epithelial cells and extra cellular matrix. So, the diverse function of the S-layers strongly reflects adaptations of lactobacilli to specific ecological and environmental conditions. The purified preparations of recombinant S- layer protein could be the stepping stone to explore these molecules as Microbe Associated Molecular Patterns (MAMPs) to understand probiotic functionality in the gut through modulation of immune response and pathogen exclusion besides nano-technological applications and antigen/vaccine carriers.

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