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S-layer proteins as immune players: Tales from pathogenic and non-pathogenic bacteria

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ABSTRACT

In bacteria, as in other microorganisms, surface compounds interact with different pattern recognition receptors expressed by host cells, which usually triggers a variety of cellular responses that result in immunomodulation. The S-layer is a two-dimensional macromolecular crystalline structure formed by (glyco)-protein subunits that covers the surface of many species of Bacteria and almost all Archaea. In Bacteria, the presence of S-layer has been described in both pathogenic and non-pathogenic strains. As surface components, special attention deserves the role that S-layer proteins (SLPs) play in the interaction of bacterial cells with humoral and cellular components of the immune system. In this sense, some differences can be predicted between pathogenic and non-pathogenic bacteria. In the first group, the S-layer constitutes an important virulence factor, which in turn makes it a potential therapeutic target. For the other group, the growing interest to understand the mechanisms of action of commensal microbiota and probiotic strains has prompted the studies of the role of the S-layer in the interaction between the host immune cells and bacteria bearing this surface structure. In this review, we aim to summarize the main latest reports and the perspectives of bacterial SLPs as immune players, focusing on those from pathogenic and commensal/probiotic most studied species.

1. Introduction

The constant fight for survival has been and still is the driving force for the evolution of all living beings that inhabit the Earth. To survive, all living organisms have had to develop their own defence tools to adapt to the environment. In bacteria, as in other microorganisms, surface molecules have played a key role in this phenomenon since they mediate the interaction of each cell with its environment. Surface compounds such as lipopolysaccharides (LPS), lipoteichoic acids, lipoproteins, glycoproteins, and flagellins are part of the so-called MAMPs (microbe-associated molecular patterns) since they are highly conserved components (Mogensen, 2009). These structures interact with different pattern recognition receptors (PRRs) expressed by host cells which usually triggers a variety of cellular responses that result in modulation of the immune system. In the last years, several surface proteins non-covalently bound to the bacterial cell wall have gained attention as MAMPs, and some PRRs such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) have been proposed or even identified as specific receptors (do Carmo et al., 2018).

Among PRRs involved in bacterial recognition, the TLRs are likely the most studied. TLRs are a family of transmembrane proteins that can be located on the cell surface (*i.e.* TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10) or in endosomes (*i.e.* TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR13) (Botos et al., 2011). After ligand recognition, TLRs recruit adaptor proteins such as MyD88 (myeloid differentiation primary response protein) and TRIF (IFN-β-inducible TIR domain-containing protein) which initiates a cascade of signal transduction that culminates in the activation of the transcription factor NF-κB, and regulation of proinflammatory cytokine expression (Kawasaki and Kawai, 2014). On the other hand, the term C-type lectin was initially introduced to

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designate a group of proteins that recognized carbohydrates (lectins) in a Ca+2-dependent manner. However, it has been shown that many C-type lectins do not bind calcium and can recognize a wide range of ligands (Brown et al., 2018). This protein family share at least one C-type lectin domain (CTLD) and the transmembrane proteins were described mainly in myeloid cells such as monocytes, macrophages, granulocytes, and dendritic cells (Sancho and Reis e Sousa, 2012; Mayer et al., 2018). The CLR signaling pathway culminates, like the TLR pathway, in the activation of the canonical transcription factor NF-κB. In consequence, the simultaneous recognition of ligands by both types of receptors can potentiate, inhibit, or modulate the immune response (Thaiss et al., 2016).

Regarding the surface components that could mediate specific interactions with host cells, the S-layer, a two-dimensional macromolecular crystalline structure formed by (glyco)-protein subunits present on the surface of prokaryotes, deserves special attention. The S-layer was discovered by Houwink in 1953 (Houwink, 1953), but only in the 1970s did it begin to attract the attention of the scientific community (Sleytr and Thorne, 1976). Given that the biomass of prokaryotic microorganisms vastly exceeds the biomass of eukaryotic organisms, and that S-layer proteins (SLPs) account for 10–15% of total cellular proteins, they could be considered as one of the most abundant biopolymers on our planet (Sleytr et al., 2014). The S-layer is ubiquitous in prokaryotes and has been described in both Archaea and Bacteria (Sára and Sleytr, 2000). In Gram-negative bacteria, it is associated with LPS (Awram and Smit, 2001), whereas in Gram-positive bacteria, it is generally associated with the peptidoglycan through secondary cell wall polymers (Mesnage et al., 2000; Sára, 2001) (Fig. 1).

One of the most notable features of S-layer is that, after isolation, its proteins are capable to form self-assembled structures in solution (Liu et al., 2017), to reassemble at solid supports (Stel et al., 2018), and to cover liposomes (Luo et al., 2019; Meng et al., 2023), nanocapsules and nanoparticles (Toca-Herrera et al., 2005; Pum and Sleytr, 2014; Huggias et al., 2020). These extraordinary properties together with their structural regularity and osmotic and mechanical stability (Toca-Herrera et al., 2004; Fioravanti et al., 2022), have driven their application to the development of innovative immobilization and vehicle systems at nanoscale for different types of molecules, including antigenic proteins, bioactive molecules, and metal nanoparticles (Sleytr et al., 2014; Klotz and Barrangou, 2018; Luo et al., 2019; Bolla et al., 2022).

In Bacteria, the presence of S-layer has been described in both pathogenic and non-pathogenic strains (Sára and Sleytr, 2000; Palomino et al., 2023; Ravi and Fioravanti, 2021). Although glycosylation is the posttranslational modification most frequently found in SLPs, the studies revealing glycan structures in these glycoproteins are scarce. In pathogenic bacteria, the structure of the glycosidic residues present in SLPs were studied in some strains of *Tannerella forsythia* (Posch et al., 2011) and *Clostridioides difficile* (Richards et al., 2018). On the other hand, among non-pathogenic bacteria, there are reports about the composition and structure of glycans in the SLPs from *Lentilactobacillus kefiri* (Cavallero et al., 2017; Malamud et al., 2020) and *Lentilactobacillus buchneri* (Anzengruber et al., 2014) strains.

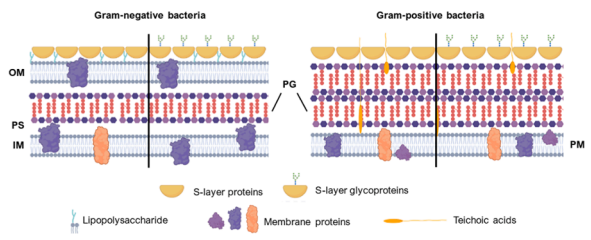

Fig. 1. Schematic illustration of the interaction of surface layer (glyco)-proteins with bacterial cell envelopes. PM: plasma membrane; PG: peptidoglycan; IM: inner membrane; PS: periplasmic space; OM: outer membrane. Created in BioRender.com.

Despite no common function to all S-layers has been described so far, among the most important functions attributed to bacterial S-layer we can mention acting as molecular sieves, binding of molecules and ions, mediating adhesion to different substrates, and protecting bacteria against various environmental factors (*i.e.* phages, reactive oxygen species, osmotic and mechanical stress, antimicrobial peptides, radiotherapy) (Gerbino et al., 2015; Farci et al., 2016). Moreover, some SLPs have the potential to act as degradative enzymes (Prado Acosta et al., 2008; Ahn et al., 2006). As surface components that mediate specific interactions with host cells, special attention deserves the role that SLPs play in the interaction of bacterial cells with humoral and cellular components of the immune system. In this sense, some differences can be expected between pathogenic and non-pathogenic bacteria. In the first group, as we will discuss later, the S-layer constitutes an important virulence factor, which in turn makes it a potential therapeutic target. For the other group, the studies of the role of the S-layer in the bacteria-host immune cells interaction have been prompted by a growing interest to understand the mechanisms of action of both commensal and probiotic strains bearing this surface structure. In this context and considering the potentiality of SLPs for the development of new adjuvants and antigenic targets, this review proposes to discuss the main latest reports and the perspectives of bacterial SLPs as immune players, focusing on those from some of the pathogenic and non-pathogenic species most studied in this field.

2. Non-pathogenic bacteria

The collection of all microbes that naturally live on and inside our body is called as “microbiome”. Nowadays, it is recognized that a balanced microbiome is fundamental for health and wellness in many ways. Consumption of probiotics, defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Hill et al., 2014), has been shown to contribute to the gut homeostasis as well as to induce beneficial effects on other organs and tissues (Akutko and Stawarski, 2021; Fang et al., 2021; Li et al., 2021). Among both commensal and probiotic microorganisms, bacteria are likely the most representative group of microbes, and in consequence the studies looking for understanding the complex host-bacteria cross-talk have been increased in the last years (Martínez-López et al., 2019; Liu et al., 2020). In this sense, the role of both the S-layer and other surface proteins in the impact of non-pathogenic bacteria on mucosal and systemic immune responses has been investigated for several research groups. In this section, we summarized the studies reported for four different bacterial species: *Lactobacillus acidophilus, Lactobacillus helveticus, Lentilactobacillus kefiri* and *Propionibacterium freudenreichii.*

2.1. *Lactobacillus acidophilus*

L. acidophilus, an indigenous member of the human gastrointestinal microbiota, is one of the most commercially significant bacterial species, and several strains are commonly used as probiotics in the dairy and dietary supplement industries worldwide (Goh et al., 2021). The surface proteome of *L. acidophilus* strains is quite complex. The S-layer is constituted by major SLPs (*i.e.* SlpA, SlpB and SlpX) and various proteins which are loosely associated with or embedded within the lattice named as S-layer associated proteins or SLAPs (Johnson et al., 2013, 2017; Klotz et al., 2020). The role of the surface proteins in the immunomodulatory properties of these bacteria has been extensively investigated in the probiotic strain NCFM and some studies with the strains ATCC 4356 and CICC 6074 were also published.

In 2008, Konstantinov and colleagues took advantage of an array of *L. acidophilus* NCFM mutants and identified the SlpA as the first probiotic bacterial ligand responsible for the binding of bacteria to DC-specific ICAM-3-grabbin non-integrin (DC-SIGN) receptor expressed on the surface of dendritic cells (DCs) and demonstrated that SlpA-DC-SIGN interaction favours the priming of T cells towards a Th2 response.

Indeed, a SlpA-knockout mutant (SlpB-dominant strain NCK1377-CI) showed not only a reduced binding to DC-SIGN but also triggered a different immune profile on DCs, characterized by higher concentrations of proinflammatory cytokines such as IL-12p70, TNF-α and IL-1β in comparison with wild-type bacteria (Konstantinov et al., 2008). Moreover, it has been shown that the oral administration of the strain NCK2187 (expressing only the *slpA* gene) or its purified SlpA protects against intestinal inflammation and dysbiosis in two different murine models of colitis (a pathogenic T-cell transfer model and a DSS-induced model) (Lightfoot et al., 2015). Additionally, NCK2187 and its purified SlpA were able to down-regulate the mucosal pro-inflammatory response in mice infected with *Citrobacter rodentium*, which contributed to accelerate the clearance of pathogenic bacteria. All these immunoregulatory effects are dependent of the interaction between SlpA and SIGNR3 (the murine ortholog of DC-SIGN) since such protection was not observed in *Signr3*$^{-/-}$ mice (Lightfoot et al., 2015). These results highlight the potential usefulness of SlpA and SlpA-expressing lactobacilli for the prevention or treatment of gut inflammatory disorders.

Similarly, pre-treatment with a 46 kDa SLP purified from *L. acidophilus* NCFM attenuated the production of IL-1β, TNF-α and reactive oxygen species (ROS) in LPS-stimulated RAW264.7 murine macrophages by the inhibition of the MAPK and NF-kB signaling pathways (Wang et al., 2018). In this sense, phosphorylation of IκBα was inhibited by SLP pre-treatment, with a consequent reduction of NF-kB p65 translocation into the nucleus. In addition, this SLP significantly downregulated the expression of inducible nitric oxide synthase (iNOS) and ciclooxigenase-2 (COX-2) (Wang et al., 2018). The same SLP also exerted an anti-inflammatory effect on TNF-α-stimulated human intestinal epithelial Caco-2 cells reducing secretion of IL-8, cell apoptosis and NF-kB p65 nuclear translocation (Wang et al., 2019). Furthermore, cell integrity and permeability were also improved after SLP stimulation by restoration of zonula occludens-1 (ZO-1) and occludin expression. Unfortunately, besides the coincidence of the apparent molecular mass, the authors did not provide additional evidence to confirm the identity of this SLP as the previously mentioned *L. acidophilus* derived SlpA. In agreement with all the results discussed above, the anti-inflammatory effect on LPS-stimulated RAW264.7 cells as well as the amelioration of DSS-induced mice colitis have been also reported for the SlpA from the strain *L. acidophilus* CICC 6074 (Cai et al., 2018).

Regarding the SLPs derived from the strain *L. acidophilus* ATCC 4356, the published reports are more focused on their antibacterial (Li et al., 2011a, 2011b; Prado Acosta et al. 2016) and antiviral activity (Martínez et al., 2012; Gao et al., 2016; Prado Acosta et al., 2019) than on their immunomodulatory ability. However, Gao and colleagues demonstrated that the inhibition of H9N2 avian influenza virus invasion into BMDCs could be attributed to the stimulation of the IFN-1 signaling pathways and to the regulation of the inflammatory response. In particular, secretion of the anti-inflammatory cytokine IL-10 by SLP-treated cells was significantly higher than secretion from BMDCs infected with only the H9N2 virus, whereas TNF-α showed an opposite trend (Gao et al., 2016). Since the invasion by H9N2 virus could be mediated by DC-SIGN, the authors hypothesize that there is a competition for binding to the receptor which leads to the inhibition of the infection as well as to the immunomodulatory effects on BMDCs. Indeed, the interaction between the SLP from *L. acidophilus* ATCC 4356 and DC-SIGN was demonstrated in a set of binding assays using murine fibroblasts expressing DC-SIGN (3T3-hDC-SIGN) (Prado Acosta et al., 2016). So far, DC-SIGN only has been proved to have carbohydrates as ligands. Unfortunately, although all the studies performed by different research groups revealing that SlpA from *L. acidophilus* exert its function through recognition by DC-SIGN or its murine ortholog, and the presence of carbohydrate residues was reported in NCFM (Konstantinov et al., 2008) and ATCC 4356 (Fina Martin et al., 2019) strains, the detailed structure of the glycans present in *L. acidophilus* SlpA was not reported up to now.

The immunomodulatory activity of some of the SLAPs from *L. acidophilus* NCFM has also been studied using mutants. For instance, the strain NCK2258 generated by deletion of *lba1029*, a putative SLAP gene, showed a reduced induction of TNF-α in murine DCs compared to wild-type strain (Johnson et al., 2013). Moreover, a mutant strain deficient in one of the most prevalent SLAPs identified in the exoproteome of the *L. acidophilus* NCFM, a 72 kDa serin protease designated PrtX, induced higher secretion of IL-6, IL-12 and IL-10 in mouse BMDCs than wild-type strain, along with some morphological changes and increased adhesion to different substrates such as mucin and fibronectin (Johnson et al., 2017). Recently, Klotz and colleagues performed bacterial/DCs co-incubation assays using deficient strains for four different SLAPs (LBA0046, LBA0864, LBA1426 and LBA1539) and observed that the absence of those proteins induce changes in cytokine production, with predominancy of an anti-inflammatory profile in comparison to the parent strain (Klotz et al., 2020). Collectively, all these results suggest that both SLPs and SLAPs could contribute to the cellular immune response triggered by direct interaction between *L. acidophilus* surface and cellular receptors.

2.2. *Lentilactobacillus kefiri*

Lentilactobacillus kefiri (formerly known as *Lactobacillus kefiri*) is a bacterial species found in different fermented dairy foods such kefir, cheese and other fermented foods but also in silage and swine intestines. Several strains have been characterized as potentially probiotic through *in vitro* and *in vivo* assays (Carasi et al., 2022). The presence of S-layer in *L. kefiri* strains isolated from kefir grains has been demonstrated by first time by Garrote and collaborators (Garrote et al., 2004), and since then, different studies have been conducted to get insight into the structural and functional properties of the SLPs isolated from different *L. kefiri* strains (Bolla et al., 2020a, 2020b; Mobili et al., 2009a, 2009b). Up to now, all the *L. kefiri* SLPs characterized showed to be glycosylated (Mobili et al., 2009b; Malamud et al., 2017), but only the composition and structure of four SLPs were described: the SLPs from the strains CIDCA 83111 (SLP-83111) and JCM 5818 (SLP-5818) showed to bear both *O*- and *N*-linked glycans, meanwhile the SLPs isolated from strains CIDCA 8321 (SLP-8321) and CIDCA 8348 (SLP-8348) revealed only the presence of *O*-linked chains of five glucose residues on average (Cavallero et al., 2017; Malamud et al., 2020).

Particularly, in the last years, we have focused part of our investigations on the study of the immunomodulatory activity of these SLPs, and the first results raised from the studies performed with SLP-8348. It was demonstrated that SLP-8348 is internalized by murine macrophages RAW264.7 in a process mediated by carbohydrate-receptor interactions since it is inhibited by glucose, mannose, or the Ca^{+2}-chelating agent EGTA (Malamud et al., 2018). Even though the SLP-8348 is not able to induce cell activation by itself, it enhances the LPS-induced response, increasing the expression of surface cell markers such as MHC-II, CD86 and CD40, as well as the expression of IL-6 and IL-10 at both transcript and protein levels in comparison with LPS-stimulated macrophages. This synergistic effect is completely abrogated in presence of EGTA (Malamud et al., 2018). Even though SLP-8321 and SLP-5818 present differences in their glycosylation patterns, a similar effect was observed on LPS-stimulated RAW264.7 cells for the three SLPs (Malamud et al., 2020).

On the other hand, the three *L. kefiri* SLPs induce activation of bone marrow-derived $CD11c^{+}$ cells (BMDCs), with secretion of IL-6 and TNF-α and upregulation of co-stimulation markers such as CD40 and CD86. Moreover, internalization and stimulation assays using BMDCs from wild-type and CLR-deficient C57BL/6 mice revealed that recognition of SLP-8348 and SLP-8321 is mediated by Mincle, while SignR3 acts as the receptor for SLP-5818 which could be expected considering the differences in the glycosidic moieties present in those proteins (Malamud et al., 2019, 2020). To note, the incubation of BMDCs with these *L. kefiri* SLPs lead to the enhancement of activation of OVA-specific $CD4^{+}$ T cells from OT-II mice, and this effect is also dependent on the presence of

macrophage-inducible C-type lectin (Mincle) for SLP-8321 and SLP-8348, and on the recognition by SignR3 for SLP-5818 (Malamud et al., 2019, 2020).

Furthermore, *in vivo* studies showed that SLP-8348 glycans are crucial for the cell-mediated immune response against this protein. In this sense, a previous mild periodate oxidation of terminal glycosidic residues of SLP-8348 reduced the secretion of IFN-γ as well as the proliferation index of CD4-T cells after *ex vivo* stimulation with the native SLP-8348 of inguinal lymph nodes from mice subcutaneously injected with the oxidized glycoprotein. Moreover, mice receiving SLP-8348 as adjuvant showed an enhanced specific cellular immune response against OVA, and the results revealed that the adjuvant capacity of SLP-8348 also depends on the biological activity of its glycans (Malamud et al., 2019). These findings encourage further studies in the way of developing new vaccine adjuvants derived from non-pathogenic microorganisms.

2.3. Lactobacillus helveticus

The strains of *L. helveticus* are extensively used in food industry, particularly as lactic acid starter cultures in fermented products, and some of them have been characterized as probiotics. The presence of S-layer in *L. helveticus* from different sources has been reported with differences in the amino acid sequence among strains (Waśko et al., 2014; Suzuki et al., 2019). The role of the S-layer proteins in the probiotic properties of *L. helveticus* has been assessed for different strains such as R0052 (Johnson-Henry et al., 2007), M92 (Beganović et al., 2011), and koumiss-isolated NS8 (Rong et al., 2015). In the last case, the authors have also shown that the SLP isolated from NS8 attenuated the LPS-induced IL-12 gene expression in RAW264.7 murine macrophages in a similar way that NS8 strain itself, however, the expression of the anti-inflammatory IL-10 was not affected by the SLP as was influenced by this *L. helveticus* strain (Rong et al., 2015).

In this regard, very interesting results emerge from the studies performed by Taverniti and colleagues. They have shown that the probiotic *L. helveticus* MIMLh5 and its isolated S-layer protein (SlpA) can exert an anti-inflammatory effect on the human intestinal epithelial cell line Caco-2 by reducing the activation of the nuclear factor κB (NF-κB) in both IL-1β-stimulated and non-stimulated conditions (Taverniti et al., 2013). Conversely, they induced the expression of tumour necrosis factor alpha (TNF-α) and cyclooxygenase 2 (COX-2) in the human monocyte-derived cell line U937 via recognition through Toll-like receptor 2 (TLR-2). In these experiments, SlpA is not able to change the expression of the anti-inflammatory cytokine IL-10, in agreement with the results previously reported for SLP of *L. helveticus* NS8 with the RAW264.7 cells. For MIMLh5 SlpA, the results obtained using murine bone marrow-derived and peritoneal cavity-isolated macrophages were similar to those obtained with human U937 cell line (Taverniti et al., 2013). In the same study, *L. helveticus* MIMLh5 depleted of the S-layer by previous treatment with 5 M LiCl showed a decreased ability to induce TNF-α and COX-2 expression in both human and murine cells (Taverniti et al., 2013).

In further studies, the same group reported that depletion of the S-layer from L. *helveticus* MIMLh5 significantly reduced its ability to induce secretion of interferon beta (IFN-β), IL-12p70 and IL-10 in murine BMDCs, whereas only a minor reduction in levels of pro-inflammatory cytokines TNF-α and IL-1β was observed (Taverniti et al., 2019). On the other hand, SlpA induced only a weak expression of IL-10, TNF-α and IL-1β genes even considering that the amount of SLP assessed was approximately 100-times greater than the S-layer covering the bacterial cells used in the same experiment. Despite its poor immunostimulatory effect on BMDCs, SlpA showed to be partially responsible for the endocytosis of *L. helveticus* MIMLh5 by these cells. The endocytosis assays performed in the presence of cytochalasin D suggest that the uptake of the S-layer-depleted bacteria is restricted, thus leading to a reduction of the IFN-β-mediated production of Th1-cytokine IL-12p70 (Taverniti et al., 2019).

To note, it was reported that *L. helveticus* SBT2171 and its isolated SLP can induce the expression of the β-defensin 2 (hBD2) in human epithelial cells (colonic Caco-2 and tongue HSC-4 cell lines). This induction is triggered by the activation of c-Jun N-terminal kinase (JNK) signaling via the interaction of the SLP with TLR-2 (Kobatake and Kabuki, 2019). Human BD2 is an antimicrobial peptide usually secreted in the gut in response to inflammation or bacterial infection that contribute to protection of the host. Interestingly, the SLP extracted from other lactobacilli (*i.e. L. helveticus* JCM1120, *L. amylovorus* JCM1126, *L. acidophilus* JCM1132, *L. buchneri* JCM1115 and *L. brevis* SBT10966) stimulate hBD2 expression in different extent, and that upregulation is dependent on JNK signaling. These results suggest that this effect could be a common feature of lactobacilli SLPs (Kobatake and Kabuki, 2019). However, the involvement of the interaction between TLR-2 and these lactobacilli SLPs, as well as the motifs interacting with TLR-2 in these SLPs, if correspond, have not been identified so far.

2.4. Propionibacterium freudenreichii

Propionibacteria are usually used as ripening starter in Emmental cheese manufacturing, and as vitamins producers. Among them, *Propionibacterium freudenreichii* is a Gram-positive, pleiomorphic, microaerophilic dairy bacterium with GRAS (Generally Regarded as Safe) and QPS status (Qualified Presumption of Safety) (Rodovalho et al., 2020), and some strains have emerged as new generation probiotics since they showed beneficial effects likely due to their anti-inflammatory activity observed both *in vitro* and *in vivo*. These benefits include the amelioration of inflammation in different experimental models such as chemical-induced colitis, gut infection, necrotizing enterocolitis in newborn mice (Foligné et al., 2010; Colliou et al., 2017; Ge et al., 2020), and chemotherapy-induced mucositis (do Carmo et al., 2020).

The first report of the presence of S-layer in a *P. freudenreichii* strain (CNRZ 722) dated from almost 30 years ago, however it does not appear to be a characteristic trait of the species since SLPs were not extracted from other strains studied in that work (Lortal et al., 1993). The role of surface extractable compounds in the immunomodulatory properties of *P. freudenreichii* strains was firstly suggested by Foligné and colleagues (Foligné et al., 2010), and since then it has been explored exhaustively through different approaches. Different proteomic techniques were used to identify five proteins non-covalently associated to the cell wall (InlA, lspA, slpE, slpA and the major surface layer protein slpB) in the strain *P. freudenreuchii* CIRM-BIA 1^T (Le Maréchal et al., 2015). The extract containing all the mentioned proteins induced the release of IL-10 and IL-6 with little or no effect on IL-12, TNF-α and IFN-γ in human peripheral blood mononuclear cells (PBMCs). Moreover, guanidine-treated *P. freudenreuchii* CIRM-BIA 1^T lost the ability to induce secretion of IL-10 and, when applied simultaneously with the pro-inflammatory *Lactococcus lactis*, the extract reduced the induction of pro-inflammatory cytokines by this bacterium. Taken together, these results suggest the involvement of the extractable surface proteins in the immunomodulatory ability of *P. freudenreuchii* CIRM-BIA 1^T (Le Maréchal et al., 2015). A multi-strain study combining genomics, transcriptomics, and surface proteomics, coupled with gene inactivation was conducted by the same group. The authors identified SlpB and SlpE as two of the surface proteins playing a key role for the induction of IL-10 in PBMCs (Deutsch et al., 2017). However, the combination with other surface components or even cytoplasmic proteins are probably responsible for the diverse anti-inflammatory properties of the *P. freudenreuchii* strains.

In other studies, do Carmo and colleagues used specific antibodies and mutation of the *slpB* gene to confirm the key role of SlpB in adhesion of *P. freudenreuchii* CIRM-BIA 129 to human intestinal epithelial cells HT-29 (do Carmo et al., 2017). Interestingly, the *slpB* mutant (CB129Δ*slpB*) failed to induce IL-10 expression in HT-29 cells as well as to reduce the expression of IL-8, IFN-α, TNF-α, TLR-4 in the LPS-stimulated enterocytes in comparison with the wild-type partner

(do Carmo et al., 2020). Moreover, CB129Δ*slpB* was not able to prevent inflammation and intestinal tissue damage in a chemotherapy-induced model of mucositis while wild-type strain does, which confirm the key functional role of SlpB in the anti-inflammatory activity of *P. freudenreuchii* strains (do Carmo et al., 2020).

On the other hand, the dihydrolipoamide acetyltransferase (DlaT), one of the major S-layer proteins found in *P. freudenreuchii* P.UF1 (strain isolated from the gut of premature infants fed human breast milk), showed a key role in the induction of Th17 cells in mice. Moreover, deletion of *dlaT* gene impairs the regulation of protective Th17 response to *Listeria monocytogenes* infection (Colliou et al., 2017). Noteworthy, it was recently revealed that glycosylation of large surface layer protein A (LspA) by protein O-mannosyltransferase 1 (Pmt1) regulates the interaction with SIGNR1 on dendritic cells (DCs), which in turn controls cellular transcriptomic and metabolomic machineries. In the context of *L. monocytogenes* infection, these programmed DCs are critical to promote a protective T cell response (Ge et al., 2020). Similarly, the interaction between glycosylated LspA and SIGNR1 protects mice against chemically induced colitis, highlighting the relevance of the recognition of surface layer component LspA as well as its adequate glycosylation in the anti-inflammatory properties of *P. freudenreuchii* (Ge et al., 2020).

3. Pathogenic bacteria

As it was mentioned above, in pathogenic bacteria the S-layer represents an important virulence factor, as it can mediate pathogen adhesion to host cells and contribute to biofilm formation, as well as interact with immune cells and to contribute to the evasion of innate and adaptive immunity mechanisms (Ravi and Fioravanti, 2021). Regarding the interaction of SLPs with immune cells and their impact on immune response, here we reviewed the main results reported for two Gram-positive pathogens (*Clostridioides difficile* and *Bacillus anthracis*), and the Gram-negative oral pathogen *Tannerella forsythia*.

3.1. Clostridioides difficile

Clostridioides difficile is a rod-shaped, obligate anaerobic, spore-forming bacterium, and the major etiological agent of cases of antibiotic-associated diarrhoea (AAD). Other risk factors associated with *C. difficile* infection include advanced age (>65 years old), use of proton pump inhibitors, long periods of hospitalization and comorbidities such as chronic kidney disease, inflammatory bowel disease and immunodeficiency among most prominent (Leffler and Lamont, 2015). Two toxins, TcdA and TcdB, are major responsible of *C difficile* virulence. The glycosyltransferase activity of them on proteins of the Rho GTPase family leads to the disruption of the actin cytoskeleton, cell death and strong inflammatory response (Just and Gerhard, 2004). A third toxin, named binary (CDT), could be produced, and although its role in the disease has not been completely established, the ability to produce CDT has been correlated with fatality rate (Gerding et al., 2014). Besides toxins, *C. difficile* express several non-toxigenic surface proteins able to be recognized by the immune system.

The presence of S-layer was reported in all the strains of *C. difficile* studied so far, and in contrast to most bacteria, it consists of two protein subunits commonly referred to as the HMW (high molecular weight) and LMW (low molecular weight) proteins, which are the result of the extracellular cleavage of their precursor SlpA by the cell-wall cysteine protease Cwp84 (Dang et al., 2010). The HMW and LMW subunits form a tightly associated non-covalent heterodimeric complex where LMW protein is located at the outermost surface of the cell (Bradshaw et al., 2018). Most of the SlpA were described as non-glycosylated proteins (Qazi et al., 2009), however, few years ago Richards and collaborators demonstrated that a *C. difficile* strain of S-layer cassette type 11 (SLCT-11) has a complex O-glycan attached to the LMW subunit of its SLP, which seems to be involved in sporulation, cell length and biofilm formation of *C. difficile* (Richards et al., 2018). More recently, other studies showed that highly virulent *C. difficile* strains express glycosylated LMW proteins (Shaw et al., 2020).

The immunogenicity of HMW and LMW proteins was demonstrated since specific antibodies against these antigens were revealed in sera of both humans and animals infected with *C. difficile* (Ní Eidhin et al., 2008; Wright et al., 2008). Moreover, it was reported that antibodies against SlpA block attachment of different *C. difficile* strains to human intestinal epithelial cells showing that these surface components are significant contributors to the host cell adhesion (Merrigan et al., 2013). These results suggest that anti-SLP specific antibodies could have a protective role in the context of *C. difficile* infection. To this respect, it was shown that patients who had a single episode of *C. difficile* diarrhoea had significantly higher anti-SLP IgM titres than patients who later developed recurrent diarrhoea. Additionally, a low anti-SLP IgM level on the third day of the first episode was an independent predictor of recurrence (25-fold increased risk) (Drudy et al., 2004). More recently, a significantly higher level of anti-SlpA IgG antibodies was observed in patients with a single episode of *C. difficile* infection compared to both patients with recurrent infection and healthy controls (Mizrahi et al., 2018).

In another study, OBrien and collaborators observed that passive immunization with rabbit anti-SlpA antibodies attenuates disease and increase survival in a lethal hamster model (O'Brien et al., 2005). Later, the same group of researchers carried out a set of experiments of active immunization with the HMW-LMW complex from *C. difficile* R13537 belonging to ribotype (RT) 001 strain, combining different doses of immunogen, routes of administration and adjuvants. Despite anti-SLP IgG in serum were detected in almost all immunized hamsters, no protection was achieved against oral challenge with the pathogen (Ní Eidhin et al., 2008). Additionally, none of the sera of immunized hamsters was able to increase phagocytosis of *C. difficile*, regardless of the adjuvant used and the anti-SLP titre (Ní Eidhin et al., 2008). Similarly, hamsters vaccinated intrarectally with recombinant SlpA from *C. difficile* strain 630 and cholera toxin showed a significantly higher level of specific IgG in serum than control animals. However, protection against *C. difficile* infection was partial without significant differences between immunized and control animals (Bruxelle et al., 2016). In contrast, SlpA-vaccinated mice with the same adjuvant and by the same route showed a significantly lower *C. difficile* count in feces at day 10 after challenge than non-vaccinated mice, which correlated with the presence of anti-SlpA IgG in serum as well as fecal anti-SlpA IgA (Bruxelle et al., 2016). These opposing results could be explained, at least partially, because hamsters are more susceptible than mice against *C. difficile* infection.

The HMW protein is highly conserved in the species, while LMW subunit shows high heterogeneity among different strains isolated from animals and humans (Calabi et al., 2001; Mccoubrey and Poxton, 2001; Ni Eidhin et al., 2006; Spigaglia et al., 2011; Dingle et al., 2013). Interestingly, recent evidence revealed that the HMW subunit is anchored to the cell wall, while the LMW subunit is exposed to the surface (Willing et al., 2015; Lanzoni-Mangutchi et al., 2022). These findings suggest that the antigenic variation of surface proteins could provide a tool to escape recognition by the immune system. To this respect, Spigaglia and colleagues reported that the LMW subunit of hypervirulent *C. difficile* RT 027 shares immunogenic properties with those of the epidemic RT 001 which contributes to support the hypothesis that this protein may have a role in the evasion of the immune response (Spigaglia et al., 2011).

Regarding the direct impact on immune cells, the study performed by Ausiello and coworkers demonstrated that the SLPs isolated from *C. difficile* C253 (C253-SLP) induced the secretion of pro-inflammatory IL-1β and IL-6 by resting monocytes, the maturation of human monocyte-derived DCs (MDDC) as well as the proliferation of allogeneic T cells (Ausiello et al., 2006). Moreover, C253-SLP also stimulated the secretion of IL-10 and IL-12p70 in MDDC and induced a mixed Th1/Th2

response in naïve T cells (Ausiello et al., 2006). Similar results were obtained with the purified SLPs from different hypervirulent and epidemic (H/E) and non-(H/E) isolates of *C. difficile* since the HMW-LMW complexes induced production of IL-1β, IL-6 and IL-10 in monocytes, and no differences were observed in cellular response triggered by isolates (Bianco et al., 2011). In another study, the HMW-LMW complex isolated from a *C. difficile* RT 001 strain induced maturation of murine BMDCs characterized by secretion of IL-12p70, IL-23, TNF-α and IL-10 as well as expression of MHC-II, CD40, CD80 and CD86 in the cell surface (Ryan et al., 2011). Interestingly, despite both subunits showed to be immunogenic, separated HMW or LMW proteins were not able to stimulate BMDCs, which suggest that the interaction between the SLP and its receptor likely requires a specific spatial conformation only present in the HMW-LMW complex. Moreover, the SLP-induced cell activation showed to be TLR4-dependent since BMDCs from C3H/HeJ TLR4$^{-/-}$ mice did not respond to HMW-LMW complex stimulation. To note, in contrast to LPS, CD14 is not required to activate NF-kB pathway downstream of TLR4 via HMW-LMW complex recognition. Furthermore, a set of experiments performed with OVA-specific CD4^{+} T cells from OT-II mice, revealed that SLP-TLR4 recognition by BMDCs is crucial to induce a mixed T helper response, characterized by a significant production of IL-17, IL-4 and IFN-γ (Ryan et al., 2011). Finally, using an antibiotic-treated murine model, these authors demonstrated that both TLR4$^{-/-}$ and MyD88$^{-/-}$ mice are more susceptible to *C. difficile* infection than wild-type, TLR2$^{-/-}$ and TRIF$^{-/-}$ mice, which constitutes very strong evidence that SLP-TLR4 engagement could be important for recognition of the pathogen and the subsequent generation of the adequate adaptive immune response for bacterial clearance (Ryan et al., 2011).

In other studies, it has been shown that SLP from the same *C. difficile* RT 001 strain induces activation of the J774A.1 murine macrophage cell line, increasing secretion of pro-inflammatory cytokines and chemokines MIP-1α, MIP-2 and MCP, as well as expression of TLR2, TLR4, CD14, MHC-II, CD80 and CD86 (Collins et al., 2014). Moreover, J774A.1 cells incubated with SLP showed a higher phagocytosis rate than unstimulated cells, and that effect was significantly reduced in presence of a p38 inhibitor (Collins et al., 2014). These results show that *C. difficile* SLP can stimulate key mechanisms in antigen presenting cells (such as DCs and macrophages) which are relevant for pathogen clearance. Regarding this, further studies revealed that the *slpA* gene of two hypervirulent strains belonging to RT 027 and 078 respectively (which were associated to more severe and recurrent infections) exhibits signatures of positive selection, mainly in LMW subunit, which correlate with their ability to activate macrophages. The SLPs from RT 027 and 078 induced a more potent response in J774A.1 murine macrophages with higher production of IL-12p40 and IL-6 than the previously studied SLP from RT 001 or 014 (two less virulent ribotypes) which may contribute to increased inflammation and further tissue damage (Lynch et al., 2017). Interestingly, the SLPs from these hypervirulent strains also induced higher levels of IL-10 secretion by macrophages, which can promote differentiation of regulatory T cells, thus impairing effector adaptive response against pathogen and allowing the bacteria to persist in the gut (Lynch et al., 2017). The induction of both pro- and anti-inflammatory mediators by the SLPs from these hypervirulent strains might sound contradictory, however, this kind of mixed response contributes to build the best scenario for the pathogen.

A study performed by Liu and colleagues demonstrated that caspase-1-dependent inflammasome plays an important role in the regulation of host immune response during *C. difficile* infection using both *in vitro* and *in vivo* models. The phagocytosis of the bacterium is required to elicit inflammasome activation in macrophages and leads to both caspase-1-mediated cleavage of pro-IL-1β and subsequent production of mature IL-1β, and pyroptotic cell death. Interestingly, it was shown that cells that have lost their membrane integrity because of infection with toxigenic *C. difficile* strains (such as VPI 10,463 and BAA1805) release not only cytosolic components but also bacterial SLPs (Liu et al., 2018). Noteworthy, further studies showed that SLPs of the non-toxigenic strain *C. difficile* CCUG 37,780 can induce inflammasome activation on THP-1 cells in a dose-dependent manner (Chen et al., 2020). These assays performed using CHO-K1 cells revealed that the SLPs bind to the cell membrane, colocalizing with the membrane raft marker caveolin-1. Moreover, cholesterol depletion by methyl-β-cyclodextrin (MβCD) in these cells reduces the association of SLPs with the cell surface as well as the inflammasome activation. This behaviour was also observed in *C. difficile*-infected THP-1 cells, where SLPs bound to cholesterol-rich microdomains were observed by confocal fluorescence microscopy (Chen et al., 2020).

3.2. *Tannerella forsythia*

Tannerella forsythia is a Gram-negative, filament-shaped, non-motile, oral anaerobe and a member of the so called "red complex" bacterial consortium of the subgingival cavity strongly implicated in periodontitis, a chronic inflammation against biofilm bacteria that lead to tooth loss (Sabet et al., 2003; Chinthamani et al., 2017). Studies performed with *T. forsythia* ATCC 43,037 revealed that its S-layer is composed of two major glycoproteins of approximately 230 and 270 kDa, known as TfsA and TfsB, respectively, substituted by the same *O*-linked complex branched glycans containing several different residues such as *N*-acetylmannosaminuronic acid, *N*-acetylmannosaminuronamide, pseudoaminic acid, galactose, xylose, glucose and fucose (Posch et al., 2011). The *T. forsythia* S-layer was identified as virulence factor several years ago since it was demonstrated that the SLPs were able to mediate adherent/invasive bacterial activities on epithelial cells and murine subcutaneous abscess formation (Sabet et al., 2003; Sakakibara et al., 2007) as well as coaggregation with other oral pathogens (Shimotahira et al., 2013), while their role in agglutination of red blood cells showed to be contradictory (Sakakibara et al., 2007). Moreover, these proteins showed to be highly immunogenic since they are recognized by specific IgG antibodies present in serum of patients with periodontitis (Yoneda et al., 2003).

Sekot and colleagues reported that an S-layer deficient mutant (*Tf* Δ*tfsAB*) induced significantly higher levels of pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-8 compared with its wild-type counterpart *T. forsythia* ATCC 43,037 on human macrophages (U938 cell line). These differences were evident at the early phase of response (3 hs of incubation) whereas were no longer observed after 24 hs of stimulation. Similarly, an increased expression of IL-8 at both 3 and 24 hs was observed on human gingival fibroblasts isolated from tissue of periodontally healthy individuals that were stimulated with *Tf* Δ*tfsAB*. All these findings suggest that the S-layer is an important virulence factor since it contributes to delay the host immune response against this oral pathogen by evading innate recognition (Sekot et al., 2011). In this sense, other authors observed that the deposition of factor C3b (which mediates the activation of complement pathway leading to the assembly of the membrane attach complex on bacterial surface) is significantly higher for the mutant *Tf* Δ*tfsAB* than for the wild-type strain ATCC 43, 073 (Shimotahira et al., 2013), showing again a contribution of the S-layer to impair the recognition by the innate immune system.

It is known that surface glycosylation in bacteria can modulate responses during pathogenesis. On this regard, Settem and colleagues performed a series of *in vitro* and *in vivo* experiments using a *wecC*-deletion mutant (called ED1) which lacks a terminal trisaccharide motif of the S-layer glycan (Settem et al., 2013). They found that ED1 mutant induces significantly higher amounts of IL-6, IL-1β, IL-23 and IL-12p40 secretion in both BMDCs and peritoneal macrophages than the wild-type strain ATTC 43,037, whereas no differences were observed regarding IL-10 secretion. Moreover, flow cytometry studies revealed that loss of SLP terminal glycans results in an increased uptake by DCs which in turn seems to lead to an increment of intracellular killing. Additionally, although both wild-type and ED1 mutant strains were able to infect BALB/cJ mice, it is noteworthy that significant periodontal

bone loss was observed only in mice infected with the *T. forsythia* wild-type strain which suggests higher virulence than ED1 mutant. In line with these results, mice infected with ED1 mutant showed an increase in the Th17 response accompanied by an increased neutrophilic infiltration in the gingival tissue. These findings indicate that S-layer glycosylation contributes to modulation of DCs effector functions and to restraining Th17 response in this periodontitis model (Settem et al., 2013). In further studies, the same research group demonstrated that the S-layer glycoproteins of *T. forsythia* ATCC 43,037 are recognized by Mincle in a Ca^{+2}-dependent manner, inducing the secretion of both pro-inflammatory (TNF-α) and anti-inflammatory (IL-10) cytokines in THP-1 derived macrophages, however Mincle-SLP interaction is not required for the phagocytic uptake of *T. forsythia* by these cells (Chinthamani et al., 2017).

3.3. *Bacillus anthracis*

B. anthracis is the Gram-positive spore-forming etiological agent of anthrax disease, an often-fatal acute disease which commonly affects livestock and wildlife animals, and more rarely, humans (Chateau et al., 2020), being considered as a Category A bioterrorist agent by the Centers for Diseases Control and Prevention in 2018. The bacterium cell is covered by one of the two S-layers, Sap (surface array protein) or EA1 (extractable antigen 1), which are mutually exclusively expressed during exponential and stationary growth phase in rich medium, respectively. This switch seems to occur also during systemic infection as both SLPs have shown to be immunogenic during human anthrax infection (Baillie et al., 2003). However, the reasons why *B. anthracis* carry out this energetically expensive process are still unclear. Despite defects in cell division were reported for *sap*-deficient mutants, deletion mutants of either SLPs are viable *in vitro* but their infective ability has never been tested. In consequence, the specific contribution of the S-layer to the virulence of *B. anthracis* remains unclear (Ravi and Fioravanti, 2021).

The studies performed by Baillie and colleagues almost twenty years ago, showed the immunogenicity of Sap and EA1 in mice and proved that the presence of either of these SLPs does not adversely affect the protective immune response induced by the essential protective immunogen PA (one of the three proteins that comprise the *B. anthracis* exotoxin) in a murine model of anthrax infection (Baillie et al., 2003). Indeed, some years later, Uchida and colleagues demonstrated that intranasal immunization with recombinant EA1 plus poly(I:C) induced production of specific antibodies in feces, saliva and serum, as well as delayed the onset of the disease and significantly decreased the mortality rate in BALB/c mice challenged with intraperitoneal injection of approximately 5×10^3 spores (Uchida et al., 2012). They also showed by fluorescence microscopy and flow cytometry that *B. anthracis* spores express EA1 at the surface, which could explain the protection provided by these SLPs against infection. Moreover, if PA (10 μg) is added to the mix of EA1 (10 μg) and poly(I:C) all immunized mice are protected from a lethal challenge with *B. anthracis* spores (Uchida et al., 2012).

On the other hand, in a set of recent experiments, vaccination of C57BL/6 mice with monomeric Sap assembly domain (Sap^{AD}) or individual Sap domains (Sap^{D1}, Sap^{D2} or Sap^{D6}) injected subcutaneously (10 μg once a week, for three weeks) in the presence of Freund's adjuvant did not protect against anthrax since 75% to 100% of the animals died within one week of infection despite showing anti-Sap IgG titres in their sera (Fioravanti et al., 2019). However, subcutaneous injections of a cocktail of five anti-Sap nanobodies (Nbs^{SAI}) (6 days course of 10 injections of 20μmicrol each) able to inhibit Sap polymerization, showed to induce protection and prevent the death of mice in the same model of infection, which strongly suggest that the S-layer disrupting activity of the Nbs on live bacteria may be critical for therapeutic efficacy (Fioravanti et al., 2019).

Additionally, *B. anthracis* encodes 22 SLAPs (which are called *Bacillus* S-layer-associated proteins or BSLs) and some of them were studied as vaccine candidates. In a recent study reported by Kumar and colleagues, the subcutaneous administration of the BSL known as BA3338 showed to improve the PA vaccine protection in BALB/c mice receiving *B. anthracis* spores via intraperitoneal injection. Combination of PA and BA3338 in aluminum hydroxide favoured a Th2-type immune response with production of IL-4 and high IgG specific titres in serum (Kumar et al., 2020). Moreover, in another set of experiments performed by Jelinski and collaborators, a cocktail of three BSLs (IsdX1, IsdX2, and Bslk; 4 μg each) in alum provided protection against a lethal challenge of intranasally inoculated *B. anthracis* spores. Similar results were obtained with a five-BSLs cocktail against inhaled spores (Jelinski et al., 2020).

All these findings show that both SLPs and BSLs are very promising targets for the development of preventing therapies against anthrax.

4. Concluding remarks and perspectives

S-layer is a regular two-dimensional quasi-crystalline cell envelope that commonly covers the surface of prokaryotes. Despite no common function was described for all S-layers, it is clear that it constitutes a very important structure since it has been conserved through evolution and is one of the most abundant biopolymers in nature. Moreover, the extraordinary physicochemical and structural properties of the SLPs make them tools with a very high potential in different areas of nanobiotechnology.

In this review we aimed to highlight the most important findings related to the role of the S-layer and the SLPs as immune players in bacteria. It is to be expected that, as surface components and due to their characteristics, SLPs behave as MAMPs. In this sense, and likely due to the considerable variation in the composition, the amino acid sequence, and the presence of glycosidic moieties (even within a same species) showed by bacterial SLPs, different innate immune receptors have been identified. Among them, different representative members of both TLR (*i.e.* TLR2 and TLR4) and CLR (*i.e.* DC-SIGN, SignR1/3 and Mincle) families have been described as receptors for SLPs from pathogenic and non-pathogenic bacteria (Table 1). As expected, the diversity of recognition patterns translates into differences in the impact that the SLP-receptor interaction has on the target cell, promoting pro- or anti-inflammatory responses depending on the case (Fig. 2). Regarding non-pathogenic bacteria, this means that the immunomodulatory activity of different SLPs as well as those of the S-layer bearing microorganisms can be exploited for the development of new vaccine adjuvants as well as of novel strategies for prevention and treatment of inflammatory diseases, respectively. On the other hand, in pathogenic bacteria, along with the impact on innate immune response, the critical role of the S-layer in virulence as well as the immunogenicity of the SLPs encourage their use as vaccine, therapeutic and diagnostic targets.

Despite of the knowledge gathered over the last decades the structure-function links continues to be an unresolved issue for these exceptional biomolecules. In this sense, the arrival of new technologies, both experimental and computational, will have a critical role to pay the debt and shed light into the rational design of novel therapeutic tools based on bacterial SLPs.

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Table 1
Cellular receptors and effects induced on targets reported for S-layer proteins from pathogenic and non-pathogenic bacteria reviewed in this work.

Strain	S-layer protein	Cellular receptor	Effect on target	Reference
L. *acidophilus* NCFM	SlpA	DC-SIGN	Anti-inflammatory on DCs	Konstantinov et al., 2008
L. *acidophilus* 4356	SlpA	DC-SIGN	Anti-inflammatory on BMDCs	Gao et al., 2016; Prado Acosta et al., 2016
L acidophilus NCK2187	SlpA	SignR3	Anti-inflammatory in mice infected with *Citrobacter rodentium*	Lightfoot et al., 2015
L. *kefiri* CIDCA 8348	SLP-8348	Mincle	Pro-inflammatory on macrophages and DCs	Malamud et al., 2018, 2019
L. *kefiri* CIDCA 8321	SLP-8321	Mincle	Pro-inflammatory on macrophages and DCs	Malamud et al., 2020
L. *kefiri* JCM 5818	SLP-5818	SignR3	Pro-inflammatory on macrophages and DCs	Malamud et al., 2020
L. *helveticus* MIMLh5	SlpA	TLR-2	Pro-inflammatory on macrophages	Taverniti et al., 2013
L. *helveticus* SBT2171	SBT2171 isolated SLP	TLR-2	Induction of hBD2 in epithelial cells	Kobatake and Kabuki, 2019
P. freundereichii P.UF1	LspA	SignR1	Anti-inflammatory on DCs; prevention of inflammation in DSS-treated mice; protection of mice from L. *monocytogenes* intestinal infection	Ge et al., 2020
C. difficile PCR-RT 001	LMW-SLP/ HMW-SLP complex	TLR-4	Pro-inflammatory on BMDCs; inflammatory response in *C. difficile* infected mice	Ryan et al., 2011
T. forsythia ATCC 43,037	TfsA and TfsB	Mincle	Pro- and anti-inflammatory on macrophages	Chinthamani et al., 2017

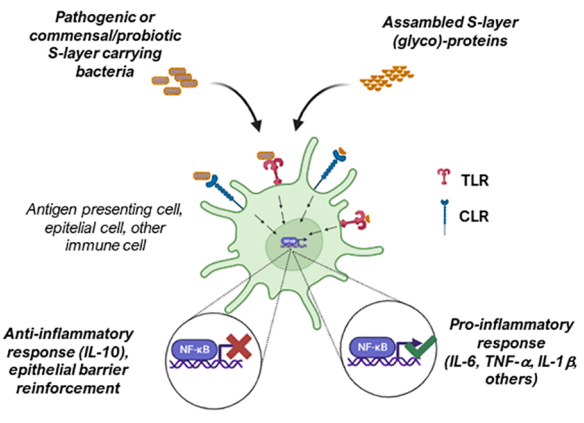

Fig. 2. S-layer covered bacteria or S-layer (glyco)-proteins interact with specific receptors expressed on the surface different cells triggering pro-or anti-inflammatory responses. TLR: Toll-like receptor; CLR: C-type lectin receptor. Created in BioRender.com.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

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