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Surface layer proteins in species of the family *Lactobacillaceae*

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Abstract
The S-layer or surface layer protein (SLP) is the most ancient biological envelope, highly conserved in several *Bacteria* and *Archaea*. In lactic acid bacteria (LAB), SLP is only found in species belonging to the *Lactobacillaceae* family, many of them considered probiotic microorganisms. New reclassification of members within the *Lactobacillaceae* family (*International Journal of Systematic and Evolutionary Microbiology*, 2020, **70**, 2782) and newly sequenced genomes demands an updated revision on SLP genes and domain organization. There is growing information concerning SLP occurrence, molecular biology, biophysical properties, and applications. Here, we focus on the prediction of *slp* genes within the *Lactobacillaceae* family, and specifically, on the neat interconnection between the two different modular SLP domain organizations and the new reclassified genera. We summarize the results in a concise tabulated manner to review the present knowledge on SLPs and discuss the most relevant and updated concepts regarding SLP sequence clustering. Our assessment is based on sequence alignments considering the new genera classification and protein domain definition with post-translational modifications. We analyse the difficulties encountered to resolve the SLPs 3D structure, describing the need for structure prediction approaches and the relation between protein structure and its anchorage mechanism to the cell wall. Finally, we enumerate new SLP applications regarding heterologous display, pathogen exclusion, immunostimulation, and metal binding.

INTRODUCTION

Lactic acid bacteria (LAB) are a varied group of Gram-positive, low GC, microaerophilic, acid-tolerant, non-spore-forming bacteria comprising five families: *Lactobacillaceae*, *Streptococcaceae*, *Enterococcaceae*, *Carnobacteriaceae*, and *Aerococcaceae*, belonging to the Phylum Firmicutes, Class Bacilli, Order Lactobacillales (Zheng et al., 2020). Coming from various plant and animal niches, some members of these families are used in food fermentation, lactic acid being the primary fermentation product (Oberg et al., 2022). Moreover, within the *Lactobacillaceae* family (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=33958), some members are generally recognized as safe (GRAS) due to their abundant appearance in food (Carasi et al., 2021; Lebeer et al., 2018; Oberg et al., 2022; Salvetti & O'Toole, 2017; Stefanovic & McAuliffe, 2019; Sun et al., 2015; Zheng et al., 2020).

Maria M. Palomino and Mariana C. Allievi contributed equally to this work and shared first authorship.

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Bacterial S-layer, found in several *Bacteria* and *Archaea*, consists of a two-dimensional self-assembling crystalline array of proteins or glycoproteins subunits, determining a semi-porous proteinaceous and the outermost component of the cellular envelope. The SLP coat exhibits a thickness between 5 and 25 nm and pore size from 2 to 8 nm, covering the rigid cell wall matrix. In Gram-positive bacteria, SLP binds non-covalently to the cell wall components, primarily peptidoglycan and other components, such as proteins, teichoic and lipoteichoic acids, and other acidic or neutral polysaccharides known as secondary cell wall polymers (SCWP) (Sleytr et al., 2014; Zhu et al., 2016). As it is the outer layer of the cell wall, SLP participates in the maintenance of the bacterial cellular shape and host interaction with the external environment, as well as acting as a molecular sieve in the exchange of nutrients and metabolites (Gerbino, Carasi, Mobili, et al., 2015; Hynönen & Palva, 2013). Here, we present updated knowledge on the SLPs found in species belonging to the *Lactobacillaceae* family.

PREDICTION OF SURFACE LAYER PROTEIN GENE

SLP from LAB has only been identified in species belonging to the *Lactobacillaceae* family, particularly in the *Lactobacillus* genus. Noteworthy, this genus has been recently split into more than 20 new genera (Zheng et al., 2020) and includes a variety of probiotic strains. Figure 1 shows the search results within the Prokaryotic Synteny & Taxonomy Explorer, SyntTax (https://archaea.i2bc.paris-saclay.fr/SyntTax/Default.aspx) (Oberto, 2013), in which the query protein is translated in the six frames using the TBLASTN algorithm and matched against the selected chromosomes of completely sequenced NCBI reference genomes available for *Lactobacillaceae* family. The predominant SLPs were selected as query proteins, such as the SlpA protein sequences from *Lactobacillus acidophilus* (Accession YP_193101.1), *Lentilactobacillus kefiri* (Accession SCA78670.1), and *Levilactobacillus brevis* (Accession ARW51672). We were able to observe a correlation between gene synteny and taxonomy (Figure 1). When phylogenetic trees were compared, based on the alignment of the 16S rRNA gene sequences and the SLP sequences, we could verify this correlation in concordance with the new classification described by Zheng et al. (2020).

Using this approach, we were able to re-define gene sequences annotated with hypothetical functions that according to synteny, are potential S-layer coding genes in *Lactobacillus* (*Lb.*), *Lentilactobacillus* (*Len.*), and *Levilactobacillus* (*Lev.*) species. Although automatic annotation has evolved, there are still
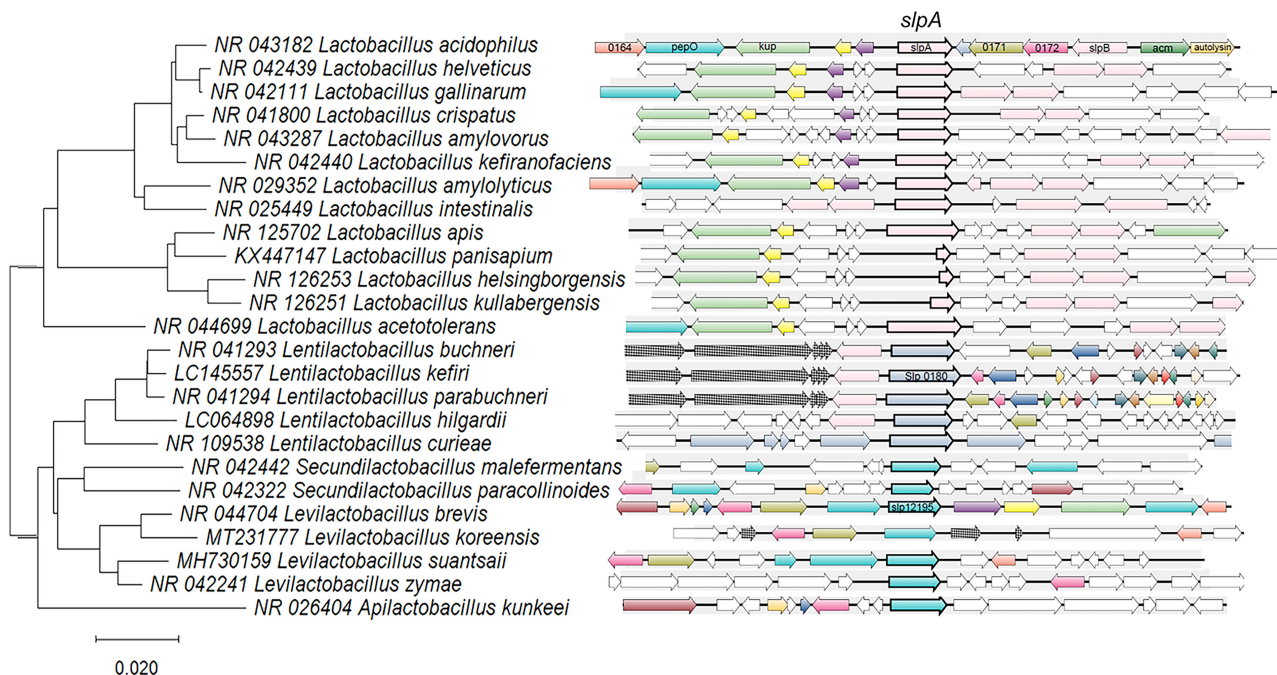
FIGURE 1 Phylogenetic alignment of the 16S rRNA and synteny. Left panel: Neighbour-joining phylogenetic tree based on concatenated alignments of 16S rRNA genes from different species, obtained using MEGA X software (Kumar et al., 2018). The tree is drawn to scale. Branch length units are the same as those of the evolutionary distances used to infer the phylogenetic relationship, and the scale bar indicates the phylogenetic distances. NCBI Accession numbers are detailed for each protein and species. Right panel: SyntTax web service results for each species. The query protein (bolded arrow) is matched against the selected chromosomes, translated in the six frames using the TBLASTN algorithm. The DNA 15,000 bp sequence segment is centred on the TBLASTN hit and translated to all the open reading frames according to GenBank annotations. Paralogues are indicated by an identical colour.

hypothetical functions that include S-layer-associated protein (SLAP) domain predictions. Specifically, annotation as a hypothetical protein was found in the case of *Lb. acetotolerans* LA749 (LA749_00955), *Lb. amylolyticus* L6 (B1745_00825), *Lb. helsingborgensis* ESL0183 (DLD54_07660), *Lb. kullabergensis* ESL0186 (DKL58_08285), and *Lb. panisapium* ESL0416 (GYM71_09165), or SLAP domain-containing proteins in *Lb. intestinalis* DSM 6629 (KBW87_00805) and *Lb. kefiranofaciens* 1207 (ICI50_01090). A high syntenic distribution could argue that those genes are, in fact, coding for SLP in those species. Synteny was found within the *Lactobacillus* genus in the following species: *Lb. crispatus*, *Lb. helveticus*, *Lb. acidophilus*, *Lb. amylovorus*, *Lb. kefiranofaciens*, *Lb. gallinarum*, *Lb. panisapium*, *Lb. intestinalis*, *Lb. apis*, *Lb. acetotolerans*, *Lb. helsingborgensis*, *Lb. kullabergensis*.

Secondary *slp* genes, *slpB*, and *slpX*, were also found in all analysed species, although not conserved (Table 1A and Figure 2). Complete genome analysis using SyntTax web service and TBLASTN normalized scores allowed to verify the presence of *slpB* and *slpX* genes. Since high sequence similarity can be found between SlpA and SlpB, a positive score was only inferred in those genomes where two different copies of the SLP protein coding gene were found, and duplicated results were discarded. According to synteny results, *slpB* and *slpX* were not predicted in all species using this approach (Table 1A). In *Lactobacillus*, we observed that *slpX* gene synteny was conserved for the species

TABLE 1 Heatmap table for *slp* genes in (A) *Lactobacillus* sp. (B) *Lentilactobacillus* and *Levilactobacillus* using SyntTax web service.

(A)	*Percent normalized TBlastN score*		
Genome	**slpA**	**slpB[a]**	**slpX**
Query protein (Accession No.)	YP_193101.1	CAA61561.1	KHE30430.1
Lactobacillus acidophilus La 14	80	88	79
Lactobacillus gallinarum HFD4	52	0	15
Lactobacillus amylovorus GRL1118	35	0	46
Lactobacillus helveticus FAM22155	33	0	15
Lactobacillus intestinalis DSM 6629	30	20	16
Lactobacillus crispatus DC21 1	28	33	50
Lactobacillus amylolyticus L6	27	0	0
Lactobacillus kefiranofaciens 1207	26	0	60
Lactobacillus panisapium ESL0416	16	0	0
Lactobacillus helsingborgensis ESL0183	16	0	0
Lactobacillus kullabergensis ESL0186	15	0	0
Lactobacillus apis ESL0185	15	0	0
Lactobacillus acetotolerans LA749	13	0	0

(B)	*Percent normalized TBlastN score*			
Genome	**slpA Lentilactobacillus**	**slpB Lentilactobacillus**	**slpA Levilactobacillus**	**slpB Levilactobacillus**
Query protein (Accession No.)	SCA78670.1	QGV24204.1	ANN49967.1	AAK84948.1
Lentilactobacillus kefiri DH5	97	84	24	20
Lentilactobacillus buchneri ATCC_4005	84	71	25	21
Lentilactobacillus parabuchneri KEM	79	69	26	21
Lentilactobacillus hilgardii LMG 07934	51	19	25	22
Lentilactobacillus curieae CCTCC M 2011381	20	28	20	23
Levilactobacillus brevis NPS QW 145	26	17	91	86
Levilactobacillus zymae LZ395	29	16	33	18
Levilactobacillus koreensis 26 25	21	17	25	17
Levilactobacillus suantsaii CBA3634	21	16	27	18

Note: Green and yellow represent the percent normalized TBlastN score for specific genes in the reference genomes analysed. Red represents the absence of gene similarity. Scores below 20% are observed for gene annotation defined as S-layer-associated protein (SLAP) domain-containing protein or hypothetical protein.

[a]Only different loci were evaluated.

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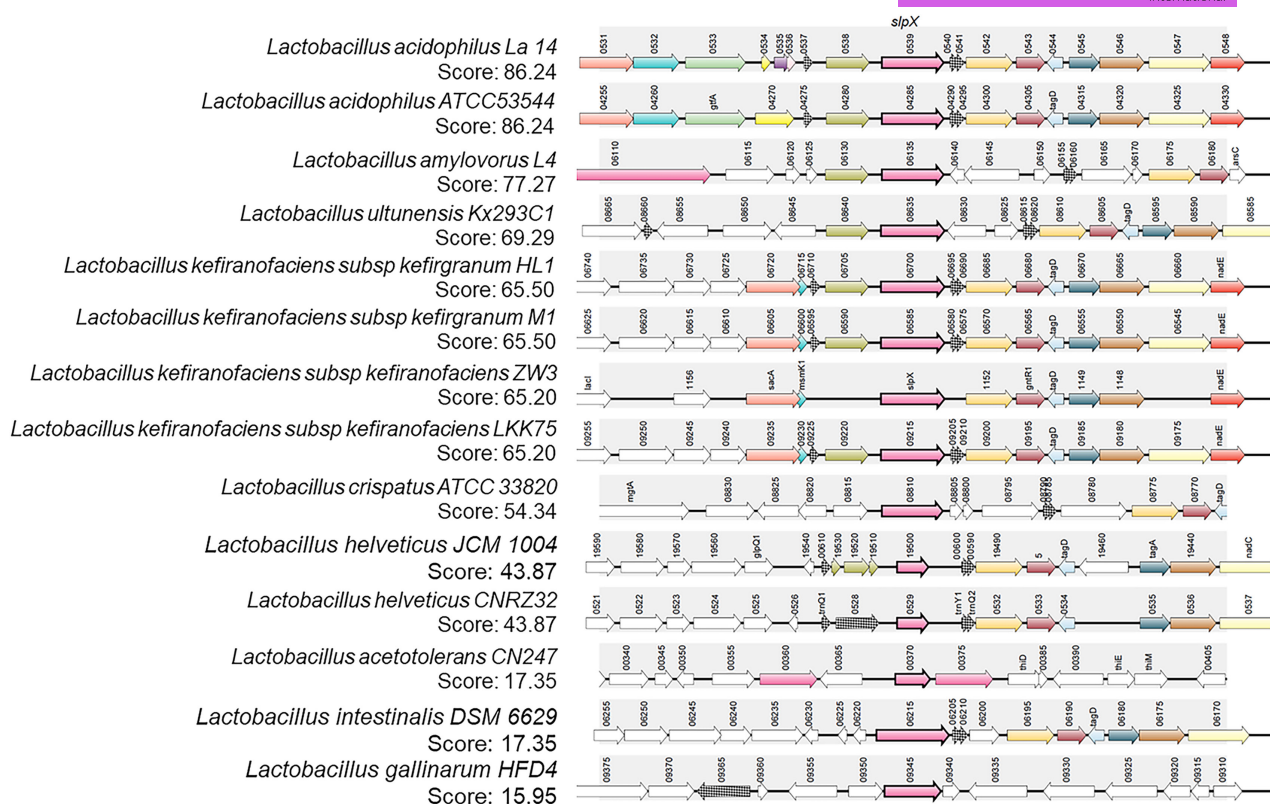
FIGURE 2 Synteny results for SlpX in *Lactobacillus*. The query protein SlpX (*Lactobacillus acidophilus*, Accession No. KHE30430.1, pink bolded arrow) is matched against the selected chromosomes translated in the six frames using the TBLASTN algorithm in SyntTax web service. The 15,000 bp DNA sequence segment is centred on the TBLASTN hit and translated to all the open reading frames according to GenBank annotations. Paralogues are indicated by identical colour. TBLASTN scores are shown.

Lb. acidophilus, *Lb. gallinarum*, *Lb. amylovorus*, *Lb. helveticus*, *Lb. intestinalis*, *Lb. crispatus*, and *Lb. kefiranofaciens* (Table 1A and Figure 2). Nevertheless, *slpB* was found to a lesser extent.

In line with these findings, Johnson et al. (2016) reported that three genes can be found within members of the *Lb. acidophilus* homology group (including *Lb. acidophilus*, *Lb. helveticus*, *Lb. crispatus*, *Lb. amylovorus*, *Lb. gallinarum*): *slp*A, *slp*B, and *slp*X, being *slp*A and *slp*B in opposite orientation to each other. Interestingly, the presence or not of secondary *slp* genes in strains of *Lb. helveticus* showed high variability by comparative genomic analyses (Waśko et al., 2014) and consequently, they could be used for strain typing in dairy products (Moser et al., 2017). Also, Fontana et al. (2019) found an increased number of *slp* genes in isolates from natural whey cultures compared to other *Lb. helveticus* strains previously reported. Genomic instability could lead to chromosomal rearrangements, such as duplications, due mainly to the presence of mobile genetic elements, such as insertion sequences (IS) (Fontana et al., 2019). Regarding *Lb. gallinarum* strains, prior genomic research indicate that they have two genes encoding SLPs: one similar to that of the genus and another that is strain-specific; however, each strain produces a single SLP, always encoded by the strain-specific

gene (Johnson et al., 2016). Although synteny between chromosomal regions was not complete among the compared strains (*Lb. crispatus*, *Lb. acidophilus*, *Lb. amylovorus*, and *Lb. helveticus*), genes were positioned in an overall syntenic organization, where the N-acetylmuramidase and autolysin/amidase are directly downstream of the genes encoding the primary SLP, *slpA* and *slpB* (Palomino et al., 2015; Johnson et al., 2016). Although some new common features could be considered signature sequences (as discussed below), confirmation of the SLP presence still relies on electron microscopy due to the differences in the predicted protein sequences for each genus. Xing et al. (2017) found four SLPs in the *Lb. kefiranofaciens* ZW3 genome isolated from traditional functional fermentation product kefir. The larger number of SLPs in this strain suggests a higher advantage for adhesion in the gastrointestinal tract (GIT).

Lentilactobacillus and *Levilactobacillus* shared the same *slpA* gene distribution concerning genetic context (Figure 1). Synteny was found in the genus *Lentilactobacillus* in the following species: *Len. buchneri*, *Len. kefiri*, *Len. parabuchneri*, *Len. curieae*, *Len. hilgardii*; and in the *Levilactobacillus* genus for *Lev. brevis*, *Lev. zymae*, *Lev. koreensis*, *Lev. suantsaii*. The main *slp* genes are presented in Table 1B. The heterogeneity distribution of *slp* genes has been previously described

in *Len. buchneri* DSM 20057 presenting at least four genes with different molecular weights (MW) as predicted products. Likewise, two complete genes and one truncated SLP gene have been identified by homology in *Lev. brevis* ATCC 367 (Makarova et al., 2006).

Although there may still be concealed sequences in the automatic gene annotation, many other genes will arise in the coming years. The genetic arrangement of the multiple SLP genes in lactobacilli is genus-dependent at some degree, and there is no genetic organization based on a consensus synteny structure. Considering this approach and based on the similarity to SLPs from *Len. kefiri* and *Lev. brevis*, we were able to find three recently sequenced species encoding S-layer genes: *Secundilactobacillus malefermentans* and *Secundilactobacillus paracollinoides* bacteria isolated from food fermentation or brewery environments (Jiang et al., 2022), and *Apilactobacillus kunkeei* an insect symbiont with similarity to *Lev. brevis* (Vergalito et al., 2020) isolated from an environmental source according to the GenBank record.

The following list of genomes do not have synteny for genes sequences that matched the SLPs used as queries: *Acetilactobacillus jinshanensis*, *Amylolactobacillus amylophilus*, *Apilactobacillus bombintestini*, *Api. kunkeei*, *Bombilactobacillus bombi*, *Companilactobacillus alimentarius*, *Co. allii*, *Co. crustorum*, *Co. farciminis*, *Co. futsaii*, *Co. ginsenosidimutans*, *Co. heilongjiangensis*, *Co. pabuli*, *Co. paralimentarius*, *Co. zhachilii*, *Fructilactobacillus fructivorans*, *Fru. sanfranciscensis*, *Furfurilactobacillus rossiae*, *Lacticaseibacillus paracasei*, *Lcb. rhamnosus*, *Lactiplantibacillus plantarum*, *Lactobacillus delbrueckii subsp bulgaricus*, *Lb. gasseri*, *Lb. gasseri*, *Lb. iners*, *Lb. jensenii*, *Lb. johnsonii*, *Lb. paragasseri*, *Lb. taiwanensis*, *Lb. terrae*, *Lapidilactobacillus dextrinicus*, *Latilactobacillus curvatus*, *Lat. sakei*, *Ligilactobacillus animalis*, *Lig. ruminis*, *Lig. salivarius*, *Limosilactobacillus fermentum*, *Lim. mucosae*, *Lim. reuteri*, *Lim. vaginalis*, *Liquorilactobacillus mali*, *Loigolactobacillus coryniformis*, *Paucilactobacillus oligofermentans*, *Schleiferilactobacillus harbinensis*.

SLP GENE EXPRESSION

SLPs can account for 10–15% of cell proteins. This high level of expression is a consequence of the presence of a strong *slp* gene promoter, but it also involves a high mRNA stability. While decay rates for mRNAs in Gram-positive bacteria for about 80% of their genes had a half-life of <7 min (Hambraeus et al., 2003), *slp* mRNA had a calculated half-life of 15 min (Boot & Pouwels, 1996, Sun et al., 2013). 5′untranslated region (5′UTR) of *slpA* mRNA can influence translation efficiency by forming a stable stem-loop structure that stabilizes the transcript and expose the ribosomal

binding site. Actually, truncation of this sequence results in a significant reduction of expression efficiency (Boot & Pouwels, 1996; Antikainen et al., 2002; Sun et al., 2013). In order to develop a high-level expression system for LAB, expression signals were identified upstream of the *slpA* gene of *Lev. brevis* and *Lb. acidophilus* and used in the low-copy-number vector pKTH2095 to express GusA and PepN reporter proteins in *Lactococcus lactis*, *Lactiplantibacillus plantarum*, and *Lb. gasseri* strains (Peterbauer et al., 2019). SLP encoding genes have been sequenced and cloned from species such as *Lev. brevis*, *Lb. acidophilus*, *Lb. helveticus*, and *Lb. crispatus*. SLP genes are preceded by more than one promoter, increasing the transcription efficiency and regulating the S-layer gene expression in response to, for instance, growth stage or environmental conditions. As one of the most abundant cellular proteins, SLP expression takes place during all stages of the bacterial growth cycle. Bacterial cells need to synthesize, translocate to the surface, and incorporate this protein into the existing S-layer lattice at high growth rates -around 500 subunits per second. At least 5×10^5 SLP subunits are needed during each cell generation when considering an average-size cell (Hynönen & Palva, 2013). Studies on the upstream *slpA* sequence of *Lev. brevis* ATCC 8287 and *Lb. acidophilus* ATCC 4356 showed that there are two subsequent promoters' sequences (P1 and P2) with no evidence of regulation during bacterial growth, at least under the conditions tested (Hynönen et al., 2014). Two promoters might offer a possibility to enhance and/or regulate gene expression. Notably, Klotz and Barrangou (2018) have recently reviewed the versatility of *slp* promoters for heterologous protein expression in various LAB hosts.

The transition from logarithmic to stationary phase produces notable SLP fluctuations (Klotz et al., 2017; Palomino et al., 2016) as well as other growth stage-dependent cell surface characteristics, like cell wall integrity. SLPs are present during all growth stages, and genes are preferentially expressed in the stationary phase since this is the most similar condition to the GIT. The stationary phase cell needs to increase *slp* expression to maintain the integrity of the cell envelope structure. The fact that a differential peptidoglycan amount is found between SLP-harbouring species and non-SLP species could explain why S-layer is required for growth in normal conditions. A decrease in peptidoglycan is observed in high salt conditions, and a consequent increase in fragility determines the need for the external highly compact S-layer component (Palomino et al., 2016). Differential SLP expression profiles have been observed for *Lb. acidophilus* ATCC 4356 in the exponential or stationary growth phase at high salt concentrations. Interestingly, *slp* knock-out mutants in *Lb. acidophilus* NCFM expressed *slpA* and *slpX* or *slpB* and *slpX* simultaneously with a differential

SLAP expression pattern (Selle et al., 2017). Also, Hynönen and Palva (2013) postulated the impossibility of creating an entirely negative S-layer mutant, indicating that at least one functional *slp* gene is essential for the growth of species carrying S-layer. Therefore, since multiple simultaneous *slp* deletion mutants were proved to be non-viable, it is difficult to assure that S-layers are essential. In fact, the presence of S-layer decreases susceptibility to mutanolysin (Valence & Lortal, 1995) and to extreme environmental conditions, including variations in pH, bile salts, proteases, and simulated gastrointestinal conditions (Eslami et al., 2013). Furthermore, removing the surface layer using lithium chloride drastically affects survival to osmotic stress or simulated gastric and intestinal conditions (Grosu-Tudor et al., 2016; Khaleghi et al., 2010; Meng et al., 2014; Palomino et al., 2016). In *Lb. acidophilus*, *slpA* expression was increased in the presence of bile salts, acidic pH, and heat stress. The induction of *slp* expression may thus take part in a general strategy to adapt and survive the harsh conditions encountered in the environment and the digestive tract (Gerbino, Carasi, Mobili, et al., 2015). This opens interesting perspectives for using S-layer as a protective coat for oral administration of unstable drug nanocarriers (Luo et al., 2019).

SLP DOMAINS ORGANIZATION

Lactobacillaceae SLPs are typically rich in basic and hydrophobic amino acids, exhibiting a generally high isoelectric point (pI) -between 9.4 and 10.4- and possess a molecular weight ranging from 35–71 kDa. In other Firmicutes, SLPs are larger and rich in acidic amino acids with a low pI. Although differences in SLP primary sequence are usually found, there are multiple similarities in terms of amino acid composition: high content of hydrophobic amino acids (31–39%), hydroxylated amino acids, serine, and threonine (23–33%), absence of cysteine residues, and the highest ratio of positively/negatively (Arg + Lys/Asp + Glu) charged residues, defining a high pI (Malamud, Bolla, et al., 2019).

We were able to distinguish a clear clustering of structural characteristics in phylogenetic trees based on deduced SLP sequences (Figure 3) by selecting good integrity representative records from the NCBI database and predicted sequences in the reference genomes. As previous studies suggested, *Lactobacillaceae* SLPs comprise two-modular regions with two essential domains: (1) the cell wall-anchoring or attachment domain and (2) the self-assembly domain (Prado-Acosta et al., 2010; Smit et al., 2001, 2002). The disposition of these domains is also characteristic of
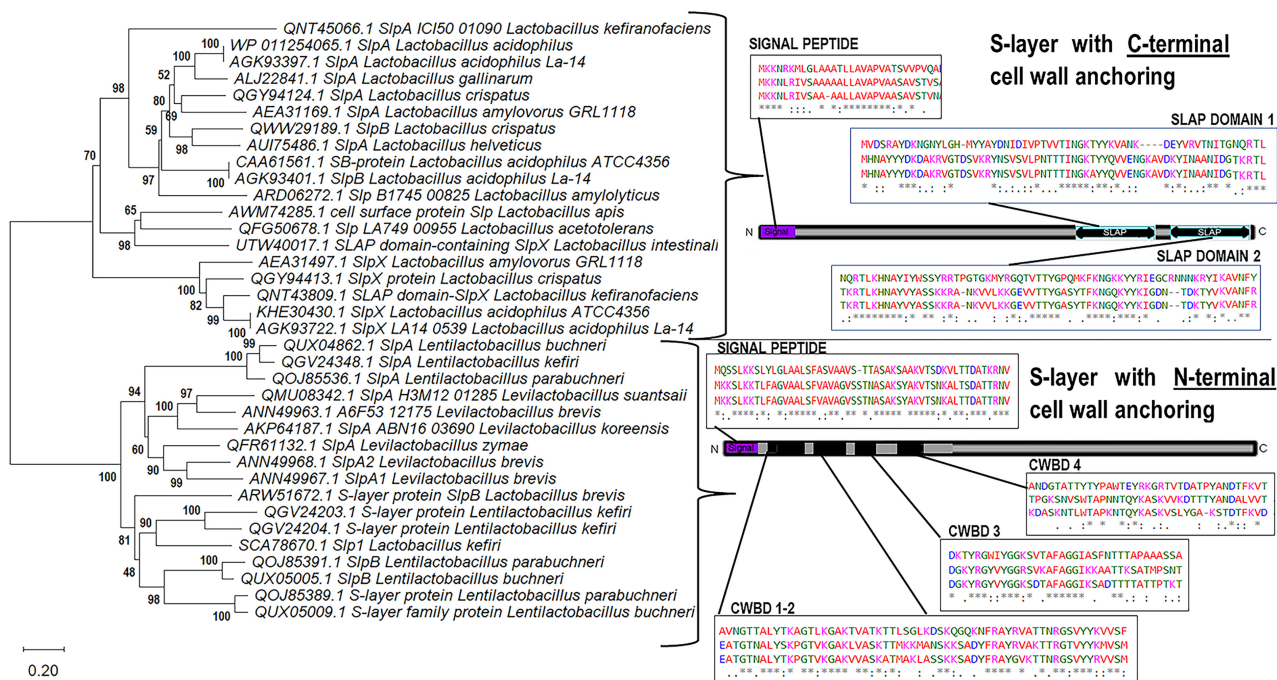
FIGURE 3 Neighbour-joining phylogenetic tree based on concatenated alignments of S-layer proteins. Neighbour-joining phylogenetic tree based on concatenated alignments of S-layer proteins from different *Lactobacillaceae* species using MEGA X software (Kumar et al., 2018). NCBI Accession numbers are detailed for each protein and species. The tree is drawn to scale. Branch length units are the same as those of the evolutionary distances used to infer the phylogenetic relationship. The scale bar indicates the phylogenetic distances expressed as the number of amino acid substitutions per sequence. Bootstrap values are indicated at the 500-replicate nodes. Grouped proteins with similar domain dispositions (braces) are represented in the sketch from N-terminal to C-terminal. Signal peptide, S-layer-associated protein (SLAP) domains or cell wall-binding domain (CWBD) are indicated in positions where the percentage of identical amino acids and conserved amino acids substitutions are higher than 50% from the sequence alignments (see Figure S1) '*'. indicates identical amino acid, ':' indicates group similarity, '.' indicates low group similarity.

different SLPs within the same species. This modular organization divides SLPs of *Lentilactobacillus* and *Levilactobacillus* species from those of *Lactobacillus*, in agreement with the new taxonomy classification (Figure 3). Although belonging to a different genus, SLP from *Lentilactobacillus* and *Levilactobacillus* shared similarity in primary sequence as deduced from BLAST scores in Table 1B. In *Lactobacillus*, the amino-terminal (N-terminal) region is involved in protein self-assembly, and the carboxy-terminal (C-terminal) region in cell wall anchoring. On the contrary, in *Levilactobacillus* and *Lentilactobacillus*, the C-terminal part is involved in self-assembly and the N-terminal part in cell wall anchoring, as detailed in Tables 2 and 3.

SLP contains a conserved N-terminal signal peptide of 25–30 amino acids (Figure 3 and Figure S1A) (Cavallero et al., 2017; Malamud et al., 2017; Palomino et al., 2016), indicative of its ability to be secreted out of the cell via the Sec-pathway, similar to other Gram-positives bacteria (Fagan & Fairweather, 2014). The signal peptide can trigger the robust secretion of target molecules and includes the A–X–A typical motif that precedes the cleavage site for type I signal peptidases commonly found in Gram-positive bacteria (van Roosmalen et al., 2004). The similarities observed in the SLP signal peptide from *Lactobacillus*, *Lentilactobacillus*, and *Levilactobacillus*

(Figure 3 and Figure S1B), correlate with the phylogenetic relationship that exists among these genera and species and their two-modular primary organization (Johnson et al., 2016; Sun et al., 2015).

The self-assembly domain was mapped for some species (Table 2). After conducting a Clustal-O alignment of *Lactobacillus* SLPs (Figure S1), we observed that the N-terminal region involved in protein self-assembly was highly variable in sequence. In contrast, the C-terminal, involved in the cell wall binding, appeared conserved between the species of the genus. The anchoring domain or cell wall-binding domain (CWBD) has been proposed for these species due to the high similarity found between the C-terminal region (Table 3) (Hynönen et al., 2014; Waśko et al., 2014). When SLPs from *Levilactobacillus* and *Lentilactobacillus* were aligned (Figure 3), it was evidenced that the more conserved N-terminal part of the protein was involved in the cell wall anchoring, while the C-terminal variable region was associated with self-assembly (Table 2). Therefore, it was possible to predict by similarity search the position of these two functional domains (self-assembly or CWBD).

SLPs act as adhesins, mediating the binding of bacteria carrying them to specific components of the extracellular matrix (ECM). The self-assembly domain

TABLE 2 Self-assembly and adhesin regions in S-layer proteins (SLPs) of *Lactobacillaceae* species.

Strain	S-layer protein	Location of Self-assembly regions (residues/total residues)	Target molecule	Reference
Lactobacillus acidophilus ATCC 4356	SlpA	N-terminal (32–238/444)	Fibronectin Collagen (I, IV) Mucin DC-SIGN	Martínez et al. (2012), Prado et al. (2016, 2019), Smit et al. (2001, 2002)
Lactobacillus acidophilus CICC 6074	SlpA	N-terminal (32–55/444) N-terminal (102–114, 170–174, 218–236/444)	HT-29 cells Membrane polar lipid liposomes	Kong et al. (2022)
Lactobacillus crispatus JCM 5810	CbsA	N-terminal (32–271/410) N-terminal (1–287, 1–274, 31–287)	Collagen (I, IV) Laminin	Antikainen et al. (2002), Sillanpää et al. (2000)
Lactobacillus helveticus CNRZ 892	SlpA	N-terminal	MIMLh5 TLR2	Sun et al. (2015)
Lactobacillus helveticus M92	SLP	Not defined	Fibronectin Laminin Collagen	Uroić et al. (2016)
Leviactobacillus brevis D6	SLP	Not defined	Fibronectin Laminin Collagen	Uroić et al. (2016)
Levilactobacillus brevis ATCC 8287	SlpA	C-terminal (179–435/435)	Fibronectin Laminin	Åvall-Jääskeläinen et al. (2008), Pum et al. (2013)
Levilactobacillus brevis ATCC 367	SlpA	C-terminal (179–435/435)	Not defined	Rykov et al. (2018)
Lentiactobacillus kefiri CIDCA 8348	SLP	Not defined	Mincle SingR3 mLangerin DC-SIGN	Malamud et al. (2018)

TABLE 3   Interactions of S-layer proteins (SLPs) of *Lactobacillaceae* species with the cell wall.

Strain	S-layer protein Accession No.	MW (kDa) pI Nº residues	Interaction site in S-layer protein (CWBD residues numbers)	Cell wall receptor	Reference
Lactobacillus acidophilus ATCC 4356	SlpA CAA61560	46.6 kDa pI: 9.59 444 aa	C-terminal 322–378 and 387–444	Wall teichoic acids Lipoteichoic acids	Fina et al. (2019), Smit and Pouwels (2002)
Lactobacillus crispatus JCM 5810	CbsA AAB58734	46.7 kDa pI: 9.69 440 aa	C-terminal 251–410 288–410	Wall teichoic acids Lipoteichoic acids Negatively charged cell wall components	Antikainen et al. (2002)
Lactobacillus crispatus K313	SlpB AFB69876	56 kDa pI: 9.48 533 aa	C-terminal 380–501	Teichoic acids	Sun et al. (2013)
Levilactobacillus brevis ATCC 8287	SlpA Q05044	48.2 kDa pI: 9.45 435 aa	N-terminal 60–90 and 165–192	Probable neutral polysaccharides	Åvall-Jääskeläinen et al. (2008)
Lentilactobacillus buchneri CD034	SlpB AFS00631	58.3 kDa pI: 9.87 558 aa	N-terminal	Lipoteichoic acids	Anzengruber et al. (2014), Bönisch et al. (2018)
Lentilactobacillus hilgardii B706	SlpLH1 CBJ17600	41 kDa pI: 9.6 417 aa	N-terminal	Cell wall	Dohm et al. (2011)

coincides in location with domains responsible for ECM binding proteins, such as collagen, fibronectin, laminin, mucin, and even to cells (Table 2). The adhesion to different cell lines has been evaluated, including Caco-2 and Intestine 407, the endothelial cell line EA-hy926, and the urinary bladder cell line T24, avian and porcine intestinal epithelial cells, HeLa cells, and HT29. The location of adhesion regions has already been revealed at the N-terminal for *Lactobacillus* (Antikainen et al., 2002; Fina et al., 2019) or C-terminal for *Lentilactobacillus* and *Levilactobacillus* (Anzengruber et al., 2014; Åvall-Jääskeläinen et al., 2008; de Leeuw et al., 2006; Vilen et al., 2009) (Table 2). This binding activity is specifically thought to mediate bacterial colonization of the gut, contributing to the probiotic's interaction with the host tissues and other factors, such as cell surface hydrophobicity, auto-aggregation, mucin- and fibronectin-binding proteins interplay. Different authors have proved that SLPs extracted from probiotic *Lactobacillus* have the ability to in vitro bind host cells and extracellular matrix proteins (Carasi et al., 2014; Prado et al., 2019; Prado-Acosta et al., 2010; Waśko et al., 2014; Zhang et al., 2017; Zhu et al., 2016).

Cell surface hydrophobicity and cell aggregation could be attributed to the S-layer. SLP removal negatively affects the adherence of *Lev. brevis* D6 and *Lb. helveticus* M92 to cells and their aggregation ability (Uroić et al., 2016). However, this was not the case for *Lb. amylovorus* (Hynönen et al., 2014). As evidenced, adhesion to epithelial cells or mucus may not be a universal feature of every SLP. Multiple S-layer sequence alignment revealed high similarity (>70%) within the *Lb. acidophilus* group in the C-terminal region (Figure S1). When compared with *Lb. amylolyticus*, an intermediate sequence similarity (approx. 40 %) was found in this region and low similarity in those presenting a N-terminal anchoring domain. In fact, previous reports have correlated differences in sequences with different immunological properties (Suzuki et al., 2019).

SLPS CELL WALL ANCHOR

Gram-positive bacteria, *Bacillus* and related genus, present S-layer homologous (SLH) domains involved in SLP cell wall anchoring (Allievi et al., 2014; Blackler et al., 2018; Janesch et al., 2013; Sleytr et al., 2014; Suhr et al., 2016). In contrast, members of the *Lactobacillaceae* species lack such motifs, presenting instead a conserved CWBD as well as negatively charged cell wall carbohydrates. These negatively charged secondary cell wall polymers (SCWP) and the highly basic amino acids in the cell wall-binding region interact via direct hydrogen bonds or electrostatic interactions, mediating the attachment to the cell wall. The interactions between SLPs and SCWP can be considered lectin-like with some degree of specificity in target recognition (Fina et al., 2019).

Conserved carbohydrate-binding motifs are detected in the cell wall-anchoring domain, consisting of high pI peptides with positively charged regions. Previous reports have evaluated the ability of these motifs to bind

the cell wall by creating truncated recombinant proteins spanning the N and C-terminal regions of SlpA (Åvall-Jääskeläinen et al., 2008; Fina et al., 2019). Two repeated amino acid sequences are predicted in the SlpA N-terminal regions of *Levilactobacillus* and C-terminal regions of *Lactobacillus* with homology to the tyrosine/phenylalanine carbohydrate-binding motifs of clostridial toxins and streptococcal glucosyltransferases (von Eichel-Streiber et al., 1992; Wren et al., 1991). These regions have also been found in the amino acid sequences of mature SlpA, positions 60–90 and 165–192 of the *Lev. brevis* N-terminal region or 322–378 and 387–444 of the *Lb. acidophilus* C-terminal region (Table 3) (Åvall-Jääskeläinen et al., 2008; Fina et al., 2019).

The C-terminal regions of SLP are particularly basic in the *Lb. acidophilus* group and related to the pfam03217 SLAP domain, recently included in the Conserved Protein Domain Family at NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=427201, created on 26-April-2021 and updated 29-September-2021). The domain is about 60 residues long and usually occurs in tandem pairs. SLAP domains are found in N-acetylmuramidase, lysis, and autolysin amidases of other species involved in the recognition of cell envelope structures. Particularly for *Lactobacillus*, they are found in species presenting SLPs with cell wall-anchoring motifs in the C-terminal region: *Lb. kefiranofaciens*, *Lb. crispatus*, *Lb. helveticus*, *Lb. amylolyticus*, *Lb. gallinarum*, *Lb. acidophilus*, *Lb. amylovorus* and *Lb. intestinalis*. Although SLAP domains are similar in size to SLH domains, there is no sequence similarity between these two motifs.

It has been experimentally confirmed that these SLAP domains are functional and required for cell wall interaction in *Lb. acidophilus* ATCC 4356 (Fina et al., 2019). Moreover, the C-terminal region of the *Lb. acidophilus* SLP has been used for heterologous display of proteins on the cell surface (Gordillo et al., 2020; Uriza et al., 2020).

The C-terminal binding domain represents one-third of the *Lb. acidophilus* SlpA protein (Table 3). This domain interacts with negatively charged SCWP and/or neutral polysaccharides (Fina et al., 2019; Sleytr et al., 2014). In Gram-positive bacteria, lipoteichoic acid (LTA) and wall teichoic acid (WTA) coexist in the cell wall; however, certain bacterial species, such as *Lacticaseibacillus casei* and *Lcb. rhamnosus*, only present LTA (Allievi et al., 2019). The LTA structures of lactic acid bacteria show high diversity among species in the length of the glycerol-phosphate chains, the degree of substitution, and the nature of the glycolipid anchor (Schneewind & Missiakas, 2014; Shiraishi et al., 2016). Additionally, glycosylation and/or D-alanine substitutions of polyglycerol phosphate in LTA display noticeable differences. The glycosylation of LTA molecules has been demonstrated in several species but not yet in *Lb. acidophilus* (Sánchez Carballo et al., 2010;

Shiraishi et al., 2016) and D-alanine substitutions have only been found in *Lcb. casei* LTA (Allievi et al., 2019; Palomino et al., 2013). It is necessary to further investigate whether the LTA glycosylation is involved in the recognition and binding of the S-layer to the cell. Fina et al. (2019) identified that the SLP C-terminus binds to LTA in the cell wall of *Lb. acidophilus* ATCC 4356 stripped from S-layer. Also, binding was inhibited in cells treated with LTA-specific antibodies or LTA extracting compounds (SDS). The highly basic nature of the SLP C-terminal portion resembles the behaviour of cationic peptides such as the well-known teichoic acid or other LTA-binding proteins (Rigden et al., 2003).

Two tandem repeats of SLAP domains are needed to interact with their substrate, offering multiple cooperative binding sites and strengthening carbohydrate attachment. The amino acid residues between 321 and 444 from the *Lactobacillus* SLP primary sequence are the minimum required for binding to glycoconjugates (Table 3). A structural model involving tyrosine residues in the interaction has already been predicted and experimentally verified (Fina et al., 2019), in agreement with consensus domain sequence in other species. Although the integrity of the sequence of the C-terminal anchoring domain seems essential, the N-terminal portion can influence the structure adopted by the mature protein and related function. As an example, *Lb. acidophilus* CP23 SlpA showed weaker immunomodulatory activity compared with *Lb. acidophilus* L-92, and even though no difference was observed in the C-terminal amino acid sequence, an insertion of an Ala–Val–Ala sequence was identified in the N-terminus of the mature protein, resulting in the accumulation of misfolded SlpA in the culture supernatant of CP23 cells (Yanagihara et al., 2015). The differences between SLPs' N-terminal primary sequence produce different results also with regard to antimicrobial activity. Previous research showed that *Lb. helveticus and Lb. acidophilus* SLP display 74% identity and 83% similarity when compared, particularly in their C-terminal region, differing mainly in the N-terminal sequence. Consequently, treatment with *Lb. helveticus* SLP resulted in a reduced ability to antagonize *Escherichia coli* and *Mycobacterium smegmatis* (Prado et al., 2016).

Recent studies in *Len. buchneri* CD034 (Bonish et al., 2018) have elucidated for the first time the structure and binding force of LTA and the S-layer O-glycosylated protein using single-molecule force microscopy and atomic force microscopy (AFM) over the protein's N-terminal region (Table 3).

SLP POST-TRANSLATIONAL MODIFICATIONS

Glycosylation is a frequent protein modification generally overlooked in bacteria. Both O- and N-linked protein

glycosylation examples have been shown in bacteria, as well as variations in the degree of glycosylation and glycan composition. Some organisms form complex S-layer lattices consisting of different protein expression profiles depending on the species and growth conditions (Palomino et al., 2016). Glycosylation has been confirmed for *Len. buchneri* (Anzengruber et al., 2014), several *Len. kefiri* strains (Cavallero et al., 2017; Malamud et al., 2020), *Lb. helveticus* and *Lb. acidophilus* (Fina et al., 2019; Konstantinov et al., 2008). However, SLP glycan structures have only been elucidated for *Len. buchneri* (Anzengruber et al., 2014) and *Len. kefiri* (Cavallero et al., 2017; Malamud et al., 2020). Glycosylation as a post-translational modification is usually indicated by the presence of two forms of SLP with mass differences (Rykov et al., 2018).

Anzengruber et al. (2014) propose a species-wide SLP O-glycosylation signature motif in *Len. buchneri* over a S-A-S-S-A-S sequence. SlpB is the most abundant protein in this species, showing O-glycosylation at four serine sites glycosylated with seven glucose (Glc) (α1-6) residues -on average- within the sequence $_{152}$S-A-S-S-A-S$_{157}$. The SLP of *Len. kefiri* CIDCA 83111 is also O-glycosylated in the signature motif S-A-S-S-A-S, with 5–8 glucose units carrying galacturonic acid (Glc5-8GalA), and another less abundant site at peptide $_{471}$T-T-T-S-A-E$_{476}$, substituted with a Glc5-8GalA2 structure. As this protein is also N-glycosylated, this was the first description of the structure of N-glycans in S-layer glycoproteins from the *Lentilactobacillus* species. Although there are 10 characteristic sequons (Asn-X-Ser/Thr) in the S-layer amino acid sequence, only two different peptides are substituted with short structures containing neutral hexoses or deoxyhexoses and amino sugars (Cavallero et al., 2017; Malamud et al., 2020). This signature motif SASSAS is located in the first 150–160 amino acid residues. However, the signature motif SASSAS in SlpE, the primary SLP of *Lev. brevis*, is located at its end (Malamud et al., 2017; Rykov et al., 2018). Whether or not *Lev. brevis* S-layer is glycosylated is still to be determined.

The glycosylation of the *Lactobacillus acidophilus* ATCC 4356 SLP was confirmed with a lectin transfer assay using Concanavalin A, a plant lectin that detects mannose residues linked to α-linked N-linked glycopeptides (Fina Martin et al., 2019). Results were consistent with previous reports in *Lb. acidophilus* and *Lb. helveticus* (Konstantinov et al., 2008). Nine possible N-glycosylation sites (Asn-X-Ser/Thr) can be predicted from the SlpA primary sequence, eight in the N-terminal and one in the C-terminal region. Since most of these sites are present in the N-terminal region, it suggests that N-glycosylation of the mature SLP would not be involved in C-terminal carbohydrate recognition; but this still needs to be confirmed.

Almost twice the amount of water is bound or coupled to a glycosylated S-layer in comparison with the non-glycosylated form (Schuster & Sleytr, 2015); however, little is known about the structure–function relationships of S-layer glycan moieties involved in providing a hydration layer. Removal of the *Lb. acidophilus* S-layer drastically affects survival to osmotic stress and modifies cell wall structure (Palomino et al., 2013, 2016). Lactobacilli are often exposed to changes in environmental osmolarity in both gastrointestinal tract and fermented foods that can compromise essential cell functions. Osmotic stress response triggers cell envelope modifications (Palomino et al., 2010, 2013; Piuri et al., 2003, 2005). In species that lack SLP, osmolarity response causes pleiotropic effects, including susceptibility to enzymatic lysis, increased sensitivity to cationic peptides, and increased capacity to form biofilm and transformation ability; these modifications result both from the changes in the peptidoglycan structure and the different zwitterion character of the lipoteichoic acid (LTA) molecule in high-salt conditions (Palomino et al., 2010, 2013; Piuri et al., 2005). Modification in cell size and shape has also been reported affecting the genomic organization, especially DNA supercoiling affecting cell division and filamentation (Piuri et al., 2003, 2005). In species with SLP increase in its synthesis is observed in the stress condition as a way to counteract the fragility of the cell wall, due to a decrease in the cell wall thickness and envelope components (Palomino et al., 2016). Since changes in the osmolarity environment impact the cell turgor pressure, and consequently, the cell volume, a high degree of glycosylation might be protective against challenging environmental conditions as shown in *Len. kefiri* (Cavallero et al., 2017). In addition, glycosylation provided a negative surface charge that might stabilize proteins in high salt conditions, creating a favourable protein–water–salt hydration network, as proposed for other S-layer glycoproteins (Schuster & Sleytr, 2015).

As demonstrated in *Len. kefiri* JCM 5818, glycosylation may also be critical for the crosstalk between bacteria carrying SLP and host cells (Prado Acosta et al., 2016). *Len. kefiri* S-layer prevents binding of *Lb. acidophilus* to C-type lectin receptors (CLR), such as the dendritic cells (DC)-specific ICAM-3-grabbing non-integrin (DC-SIGN) receptor; therefore, it shows strong activity against infection of cells expressing DC-SIGN. Deglycosylation by PNGase F remarkably reduces SLP activity, suggesting the presence of N-glycosidic chains and their involvement in the adhesion process (Prado Acosta et al., 2016). Chemical oxidation of terminal glycans is used as well to understand the role of glycans in this type of process. Treatment with meta-periodate of *Len. kefiri* CIDCA 8348 SLP results in a 50% reduction of binding to different CLR, including Mincle, SignR3, hDC-SIGN, and mLangerin. Moreover, loss of glycan integrity decreases the adjuvant capacity and immunogenicity (Malamud et al., 2018). Malamud et al. (2020) described the glycosylation pattern of three *Len. kefiri* SLPs involved in immune activation via recognition of their glycans; this is also most likely replicated in other species (Prado et al., 2021; Prado & Lepenies, 2019).

SLP STRUCTURE STUDIES

Several methods have been used for purifying SLPs, all based on disturbing the non-covalent association of SLP to the cell wall. Proteins bound to the bacterial surface are extracted using detergents through hydrogen bond breakdown with chaotropic agents (e.g., guanidine hydrochloride or urea) or by the replacement of cations (e.g., displacement of cations Na^+, Li^+, Ca^{2+}) (do Carmo et al., 2018; Sahay et al., 2015; Sleytr et al., 2014). Given SLPs' low water solubility related to their inherent self-assembly property, simple methods can be used to purify and obtain large amounts of protein after dialysis. However, a two-step procedure is needed to remove S-layer-associated proteins (Fina et al., 2019; Palomino et al., 2016), either associated with the SLP or anchored to the cell wall through non-covalent interaction domains, as described by former proteomic studies (Johnson et al., 2013). Therefore, SLP also constitutes a framework for several proteins with different functions, including host interaction (Johnson et al., 2013, 2016, 2017; Waśko et al., 2014; Zhu et al., 2016).

To date, little is known regarding the atomic structure of full-length lactobacilli SLPs. Specifically, there are several reasons why we lack three-dimensional (3D) structural information. First, SLPs' molecular weight ranges from 35 to 70 kDa, making it impossible to use nuclear magnetic resonance (NMR) structure analysis methods since they exclude molecules exceeding 30 kDa (Hynönen & Palva, 2013). Secondly, SLPs in solution form two-dimensional (2D) crystals by self-assembly instead of 3D, also presenting low solubility. Since 3D crystals are required for X-ray structure analysis, all attempts to obtain nucleation so far have been unsuccessful in screening the appropriate conditions, constantly distressed by the self-assembly of the 2D crystals (Pum & Sleytr, 2014; Sleytr et al., 2014). Finally, SLPs are usually glycosylated, influencing their secondary structure and even the stability and solubility of the whole protein (Solá & Griebenow, 2009; Szakonyi et al., 2006). SLPs secondary structure predictions are of limited value since the prediction algorithms are based on the available 3D structures of very dissimilar types of proteins. Even though no atomic resolution structure or 3D structure of any *Lactobacillaceae* full-length SLP is yet available, the structure from *Geobacillus stearothermophilus* SLP, SbsB, was obtained, showing promising results by using specific nanobodies to stabilize the SLP (Baranova et al., 2012). Substantial efforts are currently being implemented to elucidate the 3D structure by cloning small peptides (<30 kDa) and resolving them by NMR and X-ray diffraction to confirm predictions. In fact, recently (December 2022), 10 new 3D structures of *Lactobacillus* of cloned N- terminal or C-terminal domains of the S-layer protein from *Lb. amylovorus* and *Lb. acidophilus* were first resolved and released in Protein Data Bank (at https://www.rcsb.org/ with PDB Identifiers: 7QEH, 7QEC, 7QLH for *Lb. amylovorus* and 7QFG, 7QFK, 7QFL, 7QFI, 7QFJ, 7QLD, 7QLE for *Lb. acidophilus*). These very new findings will enable new insights about the residues involved in the self-assembly and cell wall anchoring in a near future.

Bioinformatic modelling is an alternative method to predict information about protein structure and identify possible binding sites. In recent work, we built a homology-based model of the SlpA C-terminal region, using as a template, the *N*-acetylmuramoyl-L-alanine amidase, Atl (Protein Data Bank (PDB) ID 4EPC) in the context of pfam03217 SLAP domain. Atl is the major murein hydrolase involved in cell separation in *Staphylococcus* and presents repeats able to bind to LTA as an anchor (Zoll et al., 2012). The structure model was presented (Fina et al., 2019), including the residues that are part of the most probable binding site (FPocket Druggability Score of 0.768) (Sosa et al., 2018) with a pocket volume of approximately 564 Å, enough to harbour two hexoses with individual volumes of 216 Å. Tyrosine fluorescence quenching was used to experimentally test the tyrosine involvement proposed to interact with carbohydrates (TYR361, TYR391, TYR393, TYR426, TYR437). Increasing concentrations of carbohydrates showed a decrease in fluorescence without changing the maximum emission and peak shape. The volume of the pocket (564 Å) supports the idea of interaction with sugar-decorated macromolecules like LTA glycosylation or glycoproteins (Fina et al., 2019). Actually, Tyrosine residues are described as ligand interactors in PDB structures 7QEH and 7QFG, probably involving the binding to the bacterial cell through interaction with LTA (Eder et al., 2019)

Other strategies are being applied to unravel the molecular structure. Electron microscopy (EM), combined with freeze-etching techniques and AFM, is employed to investigate the SLP presence in intact cells, as well as transmission electron microscopy (TEM) for the analysis of cell wall fragments. Regarding the determination of the secondary structure, most commonly used techniques like circular dichroism (CD) in the far-UV region and Fourier transform infrared spectroscopy (FITR) have been used to provide the contents of α-helix, β-sheet, and random structures on extracted SLPs (Mobili et al., 2009; Eslami et al., 2013; Lighezan et al., 2016; Meng et al., 2014; Mobarak et al., 2017).

SLPS APPLICATIONS

Heterologous display

Over the last decades, there has been increasing interest in implementing SLPs for oral vaccine development. Due to their GRAS status, adjuvant properties, and the ability to display antigenic epitopes on bacterial

surfaces, they have become excellent candidates to be used as antigen carriers. Furthermore, many lactobacilli strains are attractive vehicles for vaccine delivery since they can survive the hostile condition of the GIT (e.g., low pH, high bile concentration), colonize certain intestinal tissues, have an intrinsic adjuvant response and interact with cells of the immune system.

Recently, recombinant LAB, especially *Lactococcus* and *Lactobacillus*, have been used as a live vehicle for antigenic epitope display. Qin et al. (2014) constructed a food-grade *Lb. acidophilus* SlpA-based cell surface display vector and its feasibility was verified by the expression of green fluorescent protein (GFP) on *Lbc. casei*. Similarly, O'Flaherty and Klaenhammer (2016) could successfully express *Clostridium botulinum* and *Bacillus anthracis* antigens in the probiotic strain *Lb. acidophilus* NCFM; the engineered vaccine vector included the *Lb. acidophilus* SlpA signal peptide.

As mentioned earlier, the SLP C-terminal portion interacts with the negatively charged SCWPs and/or with neutral polysaccharides. For this reason, it has been used as an anchor for heterologous surface display of various proteins in non-genetically modified LAB. The C-terminal portion of *Lb. acidophilus* ATCC 4356 was produced in *E. coli* fused to GFP. The purified fusion protein was then bound to the surface of *Lb. acidophilus*, *Len. kefiri* and *Lb. helveticus* previously stripped with 5M LiCl (Fina et al., 2019; Gordillo et al., 2020; Uriza et al., 2020). New strategies for SLPs remotion are currently being evaluated to obtain a more compatible process with an oral vaccine platform, such as pre-growth in high salt concentration and subsequent stripping with NaCl. Moreover, protection also needs to be addressed in terms of binding stability when *Lactobacillus* cells decorated with the anchor protein are subjected to conditions mimicking the GIT (pH, high salt bile, pancreatin) (Gordillo et al., 2020). In addition, the C-terminal portion of *Lactobacillus crispatus* K2-4-3.13 SLP -SlpB- was successfully evaluated. Hu et al. (2011) evaluated the capacity of SlpB to mediate surface display by exposing GFP and β-galactosidase on the cell surface of *Lactococcus lactis* and several *Lactobacillus* species (Hu et al., 2011). Five years later, the carcinoembryonic antigen (CEA) was successfully displayed on *Lc. lactis* surface using this anchor protein. Upon oral administration, mice sera presented higher antigen-specific secretory IgA levels (Zhang et al., 2016).

In a more recent study, SLAPs domains of *Lactobacillus acidophilus* ATCC 4356 SlpA have been used as a vaccine platform to display chimeric Shiga toxin-producing *Escherichia coli* (STEC) antigens on the surface of *Lactobacillus* strains. Mice were immunized with *Lactobacillus* decorated with the fused SLAPs domains, and a STEC challenge infection was controlled efficiently (Uriza et al., 2020). Decoration of probiotic strains belonging to the *Lactobacillaceae* family, with heterologous proteins using SlpA C-terminal as

anchor domain, has turned into a promising strategy for developing of a universal platform for intestinal delivery of peptides or enzymes with therapeutic value.

Pathogen exclusion and immunostimulation

Probiotics have proven to be a good strategy for the modulation of the human intestinal and vaginal microbiota, as well as to stimulate the systemic and mucosal immune response to prevent and treat infectious diseases (De Boeck et al., 2021; Håkansson et al., 2019; Spacova et al., 2023). Several authors reported the SLP's role in antimicrobial properties and immune activation. *Lb. acidophilus* ATCC 4356 SLP C-terminal region presented murein hydrolase activity by zymogram against the cell wall of *Salmonella enterica* serovar Newport (Prado et al., 2008) and *E. coli* (Meng et al., 2015). Indeed, *Lb. acidophilus* ATCC 4356 possesses an additional mechanism against Gram-positive bacterial pathogens, such as *Staphylococcus aureus* and *Bacillus cereus*, provided by its SLP (Prado-Acosta et al., 2010). Moreover, the murein hydrolase activity of SLPs was also verified by zymogram in *Lev. brevis* and *Lb. helveticus* (Palomino et al., 2016), offering an additional survival advantage to probiotic lactobacilli over the GIT's mixed microbiota. It was shown that the SLP inhibited bacterial infection through a blockage of the specific intercellular adhesion molecule DC-SIGN (CD209) (Prado et al., 2016, 2019, 2021). Pre-treatment of pathogen bacteria with different SLPs reduces bacteria viability and also prevents infection by enterobacteria (specifically *Escherichia coli*, *Salmonella enterica* serovar *typhi*, and *Klebsiella pneumoniae*) and from *Mycobacterium smegmatis*, a non-pathogenic model for *Mycobacterium* infection. Pre-treatment with lactobacilli SLP of eukaryotic cells expressing the DC-SIGN receptor reduces their susceptibility to bacterial infections. Interestingly, glycosylation of the *Len. kefiri* S-layer is essential for attachment to the receptor and thus the inhibition of infection (Palomino et al., 2016).

Other reports have shown that the *Len. kefiri* CIDCA 8348 SLP improves the response induced by lipopolysaccharides (LPS) in macrophages in a Ca^{2+}-dependent manner; the mechanism involves an interaction between the glycosylation of the protein and the macrophage inducible Ca^{2+}-dependent lectin receptor (Mincle), a member of the C-type lectin family (Malamud, Carasi, et al., 2019). Immunostimulation is induced by SLP, evidenced by cytokine production of IFN-β, IL-12p70, and IL-10 in dendritic cells (Malamud et al., 2020; Prado et al., 2021; Taverniti et al., 2019).

Furthermore, the antiviral properties of the purified SLP of *Lb. acidophilus* ATCC 4356 has been reported to prevent the infection of Junin, Semliki Forest, Chikungunya, Zika and dengue virus in 3T3 cells overexpressing the DC-SIGN receptor (also a

member of the C-type lectin) (Martínez et al., 2012; Prado et al., 2019) and avian influenza virus H9N2 in dendritic cells (Gao et al., 2016). Additionally, SLP might suppress the inflammatory response with the inhibition of H9N2 virus infection. SLP treatment increased IL-10 expression, which aided in the control of the exacerbated inflammation caused by H9N2 infection (Gao et al., 2016). In addition, it was suggested that *Lb. acidophilus* ATCC 4356 SLP is able to interact directly with herpes simplex type 1 (HSV-1), human adenovirus type 5 (Adv-5), and vesicular stomatitis virus (VSV) (Fina et al., 2019). Even though the protein did not show veridical activity, it presents an opportunity for capturing pathogens.

SLPs metal binding

Little is known about SLP's ability to bind metals. Basically, three function roles have been attributed to the SLP in bioadsorption: as a crystalline arrangement framework, as a protective component in hostile environments, and as an ion trap.

The participation of certain ions (for example, calcium, Ca^{2+}) in the SLP self-assembly has been demonstrated by treatment effect with chelating agents (Mobarak et al., 2017). The SLP-ion interaction is unspecific and required in the specific assembly of the crystal lattice. Calcium neutralizes the SLP's carboxyl groups, reducing the protein's solubility and improving its packaging (electrostatic repulsion is avoided) (Liu et al., 2017). This lattice is essential in some physiological responses associated with the probiotic characteristic. Particularly, the immunomodulatory capacity of the *Len. kefiri* SLP, a key function in the probiotic characteristic of this strain, is affected by the presence of calcium. When exposing LPS-stimulated macrophages to *Len. kefiri* SLP, all responses of cell surface markers and cytokines increased. After the addition of a bivalent ion chelator, this increase is lost, demonstrating the central role of calcium and glycosidic residues in macrophage receptors. Biotechnologically, these results support the development of adjuvants containing SLP in its calcium conformation to stimulate antigen-presenting cells (Malamud et al., 2018; Malamud, Carasi, et al., 2019).

As already mentioned, the SLP is in direct contact with the extracellular medium, and hence, involved in bioadsorption processes. Particularly, SLP from GRAS and food-grade microorganisms, along with other components in lactobacilli (for example, cell wall polysaccharides), can retain metals in high concentrations. To address this, biosorption studies have been performed with *Len. kefiri* S-layer using FITR spectroscopy, showing that bivalent ions Cd^{2+}, Zn^{2+}, Pb^{2+}, and Ni^{2+} interact with the carboxy group

of the aspartic and glutamate side chains. This interaction introduces changes in the secondary structure of the SLP, including increased β-sheet and lower α-helix structures. The capacity of the SLP to bind these metals was quantified through Raman spectroscopy, and it was determined that Ni^{2+}, Pb^{2+}, and Cd^{2+} efficiently bind to the bacterial surface. Then, *Len. kefiri* SLP could be potentially employed to remove metals from consumption products (Gerbino, Carasi, Araujo-Andrade, et al., 2015; Malamud, Bolla, et al., 2019). Once the ability of this species to capture lead (Pb^{2+}) was demonstrated, research subsequently focused on the ability to remove this ion and the related SLP role. Pure SLP can retain lead with very high affinity, much higher than when attached to the corresponding bacteria encouraging its biotechnological use. The survival of *Len. kefiri* with or without SLP was studied in a medium supplemented with Pb^{2+}, evidencing that the SLP plays a central role in cell protection rather than ion retention. Given this biosorption property, SLP isolated from *Len. kefiri* was employed to develop platinum or silver bionanocatalysts based on SLP/polymeric nanoparticles (Bolla et al., 2020; Huggias et al., 2020).

Generally unwanted, *Len. hilgardii* is predominantly isolated in wines with a high percentage of ethanol since it produces metabolites with disagreeable organoleptic characteristics. Due to the ability of this microorganism to live in a hostile environment with high ethyl concentration, low pH, phenolic compounds, and metal ions, the species SLP proved to be essential in survival. Interestingly, copper could be retained by the SLP and was excluded from inside the cells (Dohm et al., 2011).

CONCLUDING REMARKS

SLPs have great potential due to their abundant expression, surface location, self-assembly capacity, immunostimulation, adhesion, and toxic remediation. In the same way, SLP GRAS status makes this purified protein an excellent candidate to be added to probiotic formulations, acting as a barrier for pathogens (do Carmo et al., 2018; Prado et al., 2016, 2019). Using the C-terminal region as an anchor is a suitable strategy for surface antigen display without requiring genetic modification, including oral vaccine formulations. NaCl treatment of cells could also be used to maintain GRAS status of non-genetically modified organisms (non-GMO) (Gordillo et al., 2020; Sahay et al., 2015).

The difficulties encountered in the resolution of the SLPs 3D structure have delayed the structural characterization in *Lactobacillaceae*. This is essential for gaining a deeper insight into the beneficial properties

of these proteins and for expanding the technological potential of new nano-biotechnological tools in the life and non-life sciences. A detailed glycosylation profile and the identification of cell wall interactors are also essential for advancing these developments. We expect soon to be able to obtain further knowledge on these pending facts.

AUTHOR CONTRIBUTIONS

M. Mercedes Palomino: Formal analysis (equal); funding acquisition (equal); investigation (equal); methodology (equal); writing – original draft (equal); writing – review and editing (equal). **Mariana C. Allievi:** Formal analysis (equal); funding acquisition (equal); investigation (equal); methodology (equal); writing – original draft (equal); writing – review and editing (equal). **Tania B. Gordillo:** Investigation (supporting); methodology (supporting); writing – original draft (supporting); writing – review and editing (supporting). **Sabrina S. Bockor:** Investigation (supporting); methodology (supporting); writing – original draft (supporting); writing – review and editing (supporting). **Joaquina Fina Martin:** Investigation (supporting); writing – original draft (supporting). **Sandra M. Ruzal:** Conceptualization (lead); formal analysis (lead); funding acquisition (equal); investigation (lead); methodology (lead); writing – original draft (lead); writing – review and editing (lead).

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CONFLICT OF INTEREST

The authors declare that this review was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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