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Minireview

The role of the *Listeria monocytogenes* surfactome in biofilm formation

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Summary

***Listeria monocytogenes* is a highly pathogenic foodborne bacterium that is ubiquitous in the natural environment and capable of forming persistent biofilms in food processing environments. This species has a rich repertoire of surface structures that enable it to survive, adapt and persist in various environments and promote biofilm formation. We review current understanding and advances on how *L. monocytogenes* organizes its surface for biofilm formation on surfaces associated with food processing settings, because they may be an important target for development of novel antibiofilm compounds. A synthesis of the current knowledge on the role of *Listeria* surfactome, comprising peptidoglycan, teichoic acids and cell wall proteins, during biofilm formation on abiotic surfaces is provided. We consider indications gained from genome-wide studies and discuss surfactome structures with established mechanistic aspects in biofilm formation. Additionally, we look at the analogies to the species *L. innocua*, which is closely related to *L. monocytogenes* and often used as its model (surrogate) organism.**

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Introduction

Listeria monocytogenes is a foodborne pathogen found in soil, water, plants and animals. From these sources, it enters and prospers in agroecosystems, thus contaminating food surfaces (EFSA, 2018a). High-risk products for contamination with *L. monocytogenes* are meat- and vegetable-based ready-to-eat food products, dairy products and seafood (Desai *et al*., 2019). Although ingestion of contaminated food usually causes mild to severe gastroenteritis, the invasive form of the disease often develops in high-risk individuals, the most vulnerable of which are the elderly, immunocompromised individuals, pregnant women and foetuses (Radoshevich and Cossart, 2018). The illness caused by *L. monocytogenes* is called listeriosis, and it is proportionally the leading cause of hospitalizations and death among zoonoses cases in the EU (EFSA, 2018b). Worldwide listeriosis is a relatively rare disease; however, according to the World Health Organization, the high death rate makes it a significant public health concern (CAC/GL61 2007). Strict food regulations regarding *L. monocytogenes* in ready-to-eat food products have been established in many countries, and all strains belonging to this species are currently considered virulent.

There are four evolutionary lineages of *L. monocytogenes* (I, II, III and IV) and 15 different serotypes (Liu, 2006; Orsi *et al*., 2011). Serotype 4b strains from lineage I are associated with the majority of human cases, whereas the serotypes 1/2a and 1/2c from lineage II are most frequently isolated from food (Lomonaco *et al*., 2015). On a genetic level, there are 156 different multilocus sequence typing sequence types, which are organized into 63 different clonal complexes (CC) and several singletons. CC1, CC2 and CC6 are associated with clinical cases, whereas CC9 and CC121 are hypovirulent food-related isolates (Maury *et al*., 2016). This hypovirulence is correlated with the presence of a defective gene for internalin A (*inlA*), a cell wall protein that is present in many serotype 1/2a strains and is

crucial for invading epithelial cells (Nightingale *et al*., 2005; Maury *et al*., 2016). The virulence of serotype 4b strains was found to be associated with full-length *inlA*, pathogenicity island LIPI-3 and gene clusters for teichoic acid synthesis; the hypervirulent lineage II CC4 additionally carries LIPI-4, which encodes a putative cellobiose-type phosphotransferase system (Maury *et al*., 2016).

Generally, *L. monocytogenes* strains survive extreme environmental stress that is often employed to inhibit or eradicate microbial loads in foods, including low pH, high osmolarity and low water activity (Conner *et al*., 1990; Nolan *et al*., 1992; Shabala *et al*., 2008). These strains grow in wide temperature ranges, from physiological to refrigeration temperatures and can even survive freezing at −18 °C (Walker *et al*., 1990; Miladi *et al*., 2008). Motility is an important temperature-dependent phenotypic trait of *L. monocytogenes*, as major flagellin protein FlaA (Lmo0690) levels are high at temperatures below 30 °C and decrease at physiological temperatures of 37 °C (Way *et al*., 2004). Besides stress resistance, biofilms have been suggested to play an important role in surviving the food processing environment in which *Listeria* spp. persist for months or even years (Lappi *et al*., 2004). For the survival and adaptation under various environments, *Listeria* probably relies on its rich repertoire of surface structures (Bierne and Cossart, 2007). Their role has been well described in the pathogenicity of *Listeria*, but understanding of mechanistic aspects on how these structures affect the biofilm formation process is still limited. This review provides a synthesis of the current knowledge on the effect of individual surfactome components on the biofilm formation on abiotic surfaces. We use the term surfactome to describe the whole spectrum of molecules found at the surface of *L. monocytogenes* cell wall: peptidoglycan, teichoic acids and cell wall proteins. Because surfactome components have been proven to affect *L. monocytogenes* ability to form biofilm, they may serve as the novel antimicrobial targets affecting directly the biofilm formation as an important *Listeria* persistence mechanism. This knowledge can also pave the way for identification of novel antimicrobials in the fight against the increasing occurrence of antimicrobial resistance in gram-positive bacteria.

We consider here also the analogies to the species *L. innocua*, which is closely related to *L. monocytogenes,* and they also coinhabit the food processing environments (Lappi *et al*., 2004; Milillo *et al*., 2012; Costa *et al*., 2018). Both species are tolerant to extreme environmental conditions and form biofilms, but unlike *L. monocytogenes, L. innocua* does not have virulence factors and is thus considered non-pathogenic (Glaser *et al*., 2001; Costa *et al*., 2018). Therefore, *L. innocua* is being tested as a surrogate organism for *L. monocytogenes*, especially for determining the efficiency of antimicrobial and antibiofilm strategies against *L. monocytogenes* (Sommers *et al*., 2009; Mohan *et al*., 2019).

Listeria monocytogenes biofilms

Biofilm formation process starts by adhesion of planktonic bacteria to the abiotic surface followed by proliferation and formation of microcolonies (Renier *et al*., 2011). They grow and form mature biofilms being highly organized in *L. monocytogenes,* mostly in the form of a monolayer, but several strains were found to form three-dimensional structures (Rieu *et al*., 2008). *In vitro Listeria* biofilms are not as extensive as observed for some other bacterial species, and the biofilm biomass quantified by crystal violet is usually congruent with the number of viable bacteria. The extracellular matrix of the biofilm is composed of DNA, proteins and teichoic acids (Colagiorgi *et al*., 2017). *Listeria* is able to adhere to various types of abiotic surfaces, and it attaches very firmly (Da Silva and De Martinis, 2013; Reis-Teixeira *et al*., 2017). It is believed that *Listeria* is mostly present in complex biofilms in natural environments (Sasahara and Zottola, 1993). The biofilm-forming ability of *L. monocytogenes* seems to differ between strains, which can be classified as weak, moderate and strong biofilm producers (Harvey *et al*., 2007). In a study on 98 clinical and food, *L. monocytogenes* isolates belonging to serotype groups 1/2a, 1/2b and 4b, the authors could not establish any correlation between the ability to form biofilms and serotype (Doijad *et al*., 2015). Additionally, Di Bonaventura *et al*. (2008) could not find significant differences in biofilm formation with regard to genetic lineage, source (environmental vs food) or food type (fish vs meat), regardless of the temperature and surface tested. This could also be due to differences in total fatty acid composition or hydrophobicity being strain-specific properties correlating to the biofilm-forming ability of the strain (Doijad *et al*., 2015). The known molecular determinants involved in *Listeria* biofilm formation include components of information pathways, signal transduction systems and surface structures (Renier *et al*., 2011).

The use of *L. innocua* as a surrogate organism in *L. monocytogenes* biofilm research has already been tested, but with conflicting results. Costa *et al*. (2018) found no difference between the biofilms of *L. innocua* and *L. monocytogenes* strains isolated from the same Gorgonzola factory, and these biofilms did not differ significantly in their susceptibility to the sanitizers tested. *L. monocytogenes* F6900 (serotype 1/2a) adhered to stainless steel to a larger extent than *L. innocua* ATCC 33090; however, after 72 h, no differences were found between the mature biofilms (Koo *et al*., 2014). And when grown as mixed biofilm on metal surfaces at 37 °C, *L. innocua* was shown to outgrow *L. monocytogenes*,

implying they could be competitors in their natural environment (Koo *et al*., 2014).

Surface structures involved in biofilm development

Multiple indications on the potential molecular determinants affecting biofilm formation in *Listeria* provide results of four independent transposon mutant library screens (Chang *et al*., 2012; Ouyang *et al*., 2012; Alonso *et al*., 2014; Piercey *et al*., 2016). Background strains in these studies belonged to serotype 1/2a or 4b, and libraries were screened for their biofilm phenotype at 15, 32, 35 and 37 °C (S1 Table). They infer that inactivation of proteins belonging to COGs (clusters of orthologous groups of proteins) cell envelope and cellular processes, information pathways and intermediary metabolism, affect biofilm formation. The enriched terms in the network of proteins affecting biofilm phenotype (S1 Table) are associated with intermediary metabolism (purine and other nucleobases metabolic processes, phosphorous associated metabolic processes) and carbohydrate derivative biosynthetic process. Description of interaction network analysis is provided in Appendix S1. Proteins associated with intermediary metabolism, for example metabolism of nucleotides and lipids, the citric acid cycle and membrane energetics were found to impair biofilm development only when a transposon mutant library was screened at 35 or 37 °C suggesting that these COGs may be more important at higher temperatures usually not relevant in food industry, though significant for *Listeria* virulence (Ouyang *et al*., 2012; Alonso *et al*., 2014). Eight proteins have been found to decrease biofilm formation regardless of the screening temperature or background strain serotype. They are functionally involved in peptidoglycan assembly (Lmo2229), lipoteichoic acid (LTA) synthesis (DltB, Lmo2554, Lmo0644) the flagellar apparatus (FlaA, FliP, Lmo0707), or have a yet unknown function (Lmo2056) (Fig. 1, Table 1) (Abachin *et al*., 2002; Bigot *et al*., 2005; Zawadzka-Skomiał *et al*., 2006; Webb *et al*., 2009). Transposon mutant screens hence point to surface carbohydrate synthesis associated proteins as relevant to biofilm phenotype in *Listeria* as they are enriched in the network of potential molecular determinants of biofilm phenotype, and they affect biofilm phenotype regardless of the serotype and temperature. These findings, however, remain to be validated as described later. Protein Lmo2056 has not yet been characterized, but according to protein sequence analysis, it may be an extracellular protein and contains a signal peptide and cAMP receptor protein domain found in many secretory prokaryotic proteins (Table S1).

Studies mostly focus on screening for impaired biofilm formation, whereas Piercey *et al*. (2016) reported also transposon mutants that exhibit enhanced biofilm phenotypes; these included surface proteins (muramidase MurA and flagellin A) and virulence factors (amidase Ami and internalins A, B and H). MurA is an autolysin, and its interruption results in formation of prolonged cells at 25 °C that sediment in planktonic culture leading to conclusion that the observed phenotype could be caused by excess biomass and not biofilm itself (Piercey *et al*., 2016). Mechanisms behind enhanced biofilm phenotype of the remaining transposon mutants are unknown, and may not be explained only by mediating interactions with abiotic surface or between cells like observed in *Streptococcus gordonii*. The deletion of the *S. gordonii* surface protein SspAB (involved in adhesion) enhanced the ability for biofilm formation because of inhibited histidine kinase signalling, which results in the upregulation of other adhesins, thus over-compensating the lacking adhesin to maintain the ability to form biofilms (Hall *et al*., 2019).

Cell envelope carbohydrates

Surface carbohydrates are synthesized in pathways consisting of proteins located at the surface, at the membrane and in the cytosol. Here, we will discuss the enzymes from the whole synthesis pathways when relevant, because they affect the properties of the surfactome, directly or indirectly, by their activity. The most abundant structure in the cell wall of *Listeria* is peptidoglycan, which is composed of chains of alternating units of the disaccharides N-acetylmuramic acid and N-acetylglucosamine crosslinked by muropeptides. Peptidoglycan monomeric precursors are synthesized in the cytoplasm and are flipped across the membrane in the form of lipid-anchored disaccharide–pentapeptide monomers (Fig 1A). These subunits are assembled by multimodular penicillin-binding proteins (PBPs) at the cell surface or by the recently identified SEDS (shape, elongation, division and sporulation) protein family (Guinane *et al*., 2006; Meeske *et al*., 2016). Five different PBPs belonging to class A and B have been described in *Listeria*. The PBP4 from class A (*lmo2229*) has been indicated by transposon mutagenesis to play a role in biofilm formation (Zawadzka-Skomiał *et al*., 2006; Chang *et al*., 2012; Ouyang *et al*., 2012). The interruption of *lmo2229* led to decreased biofilm formation ability on polystyrene at 32 and 37 °C in *L. monocytogenes* ScottA (serotype 4b, CC2) and 10403S (serotype 1/2a, CC7) (Chang *et al*., 2012; Ouyang *et al*., 2012). But, this may not be due to the changes in peptidoglycan, because the phenotype of the *lmo2229* mutant on the background strain EGD-e (serotype 1/2b, CC9) does not differ in peptidoglycan composition compared with the wild type (Zawadzka-Skomiał *et al*., 2006). PBP4 could be thus

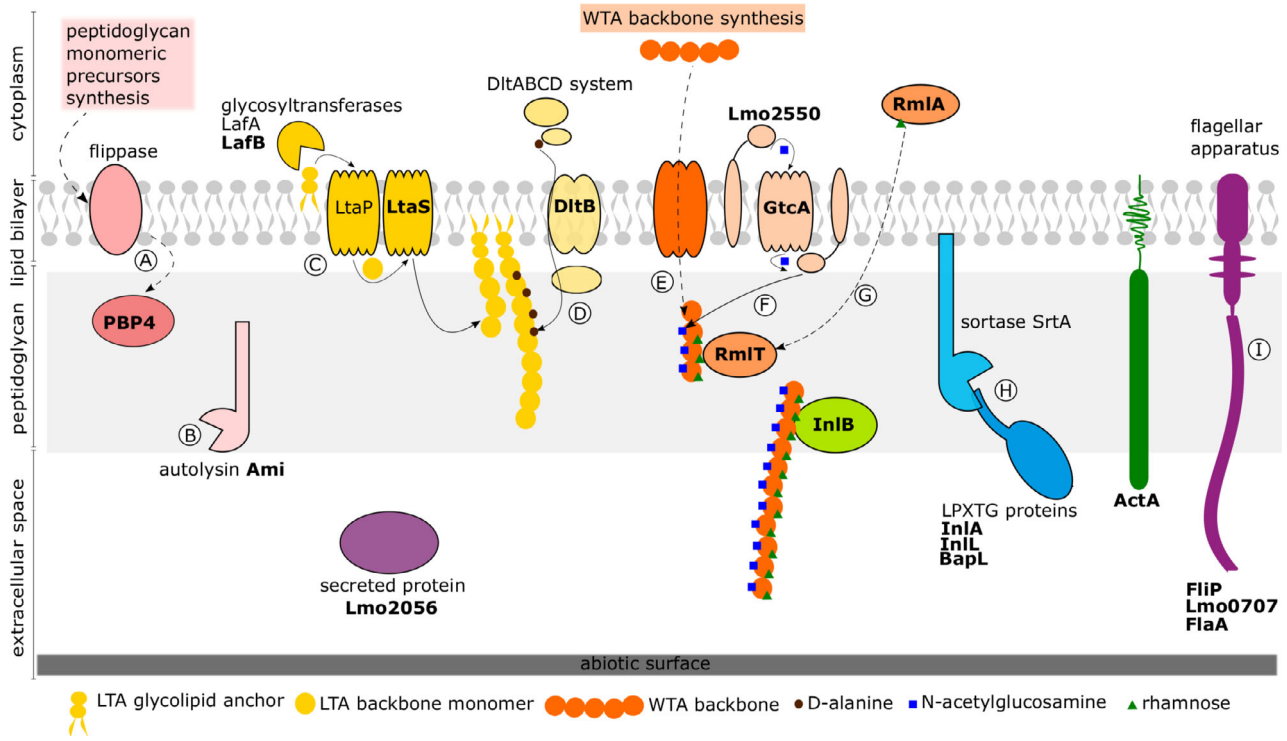

Fig. 1. A schematic diagram of the *Listeria monocytogenes* surfactome and its structures affecting biofilm formation on abiotic surface. Surfactome is composed from carbohydrates (peptidoglycan, lipoteichoic acids – LTA and wall teichoic acids – WTA), cell wall proteins and part of the flagellar apparatus. These structures are located at the surface, but the metabolism of these components is taking place in the cytosol, at the membrane and on the surface, and when relevant we consider all enzymes affecting surface structures, directly or indirectly, by their activity. The structures in bold are involved in biofilm formation and were either validated for their role in biofilm formation or their role was indicated by two or more transposon mutagenesis library screens or both. The dashed line (- - - -) is used to indicate either multistep synthesis procedure that is not completely presented here or the synthesis steps are unknown.
The peptidoglycan synthesis (A) starts in the cytoplasm and continues at the cell surface after monomers are flipped across the membrane, the assembly of peptidoglycan is carried out by multimodular penicillin-binding proteins (PBP). Peptidoglycan turnover (B) takes place directly at the cell wall by multiple autolysins. The glycolipid and the LTA backbone are synthesized by multiple enzyme system (C) and decorated by galactose (not shown) and D-alanine (D) using DltABCD system. Currently, we have limited data on WTA backbone synthesis (E), but WTA glycosylation of serotype 1/2b by N-acetylglucosamine (F) and rhamnose (G) is known and its absence negatively affects biofilm formation. The rhamnose on WTA is needed in serotype 1/2a for proper internalin B (InlB) positioning at the cell wall and its full function. Other internalins belong to the group of LPXTG proteins that are covalently bound to the peptidoglycan by sortase A via LPXTG motif (H). They are the most abundant proteins in the cell wall of *Listeria*. Flagellar apparatus (I) is the structure spanning the cell membrane and cell wall enabling *Listeria* motility at temperatures below 30 °C.

affecting biofilm phenotype by its presence at the cell wall (being part of surface proteome) mediating interactions with abiotic surface or between bacteria. Adaptations in peptidoglycan during growth and environmental changes are carried out by multiple hydrolytic enzymes (autolysins) (Fig 1B); they are non-covalently bound to the cell wall by GW modules as described later. Autolysin amidase Ami associates to the cell wall by rhamnose in serotype 1/2 and is important also for virulence of *L. monocytogenes* (Carvalho *et al.*, 2014; Carvalho *et al.*, 2018). The mutant strain lacking the Ami protein did not adhere and form biofilm as efficiently on polystyrene at 30 °C as the wild-type EGD-e strain (Kumar *et al.*, 2009). But, its overexpression caused by growth in medium with glucose and leucine, resulted in enhanced adhesion to stainless steel at 37 °C thus confirming its role in adhesion to abiotic surfaces by a yet unknown mechanism (Skovager *et al.*, 2013). Autolysins amidases Atl were shown to play an important role in adhesion to abiotic surfaces also in *S. aureus* and *S. epidermidis* (Biswas *et al.*, 2006). In *S. epidermidis* was also shown that Atl plays an active role in formation of exogenic DNA thereby increasing the initial adhesion and does not affect adhesion only by its presence (Qin *et al.*, 2007). The other well-known autolysin p60 (*iap*) of *L. monocytogenes*, which associated with the cell wall by a LysM domain, did not influence adhesion or biofilm formation on polystyrene at 30 °C (Kumar *et al.*, 2009).

Cell envelope protein expression, including several genes for PBPs and proteins with putative cell wall-hydrolysing activity, is regulated also by accessory gene

Table 1. Surfactome components involved in *Listeria monocytogenes* biofilm development on abiotic surfaces.

Protein affecting biofilm phenotype	Mechanism governing the biofilm phenotype	Primary biological role the protein plays	Homologues in *L. innocua*	Reference
PBP4 (Lmo2229)	unknown	peptidoglycan synthesis and turnover	Lin2331	Chang *et al*. (2012) Ouyang *et al*. (2012)
Ami (Lmo2558)	decreased adhesion to abiotic surface	peptidoglycan synthesis and turnover	Lin2703	Kumar *et al*. (2009)
LafB (Lmo2554)	unknown	lipoteihoic acid synthesis	Lin2699	Chang *et al*. (2012) Ouyang *et al*. (2012) Alonso *et al*. (2014)
LtaS (Lmo0644)	unknown	lipoteihoic acid synthesis	Lin0647	Chang *et al*. (2012) Ouyang *et al*. (2012) Alonso *et al*. (2014)
DltB (Lmo0973)	unknown	lipoteihoic acid synthesis (D-alanylation)	Lin0971	Alonso *et al*. (2014) Piercey *et al*. (2016)
Lmo2056	unknown	secreted protein with unknown function	Lin2162	Chang *et al*. (2012) Ouyang *et al*. (2012)
Lmo2550	decreased adhesion to abiotic surface	wall teichoic acid glycosylation	Lin2695	Brauge *et al*. (2018)
GtcA (Lmo2549)	decreased adhesion to abiotic surface	wall teichoic acid glycosylation	Lin2694	Brauge *et al*. (2018)
RmlA (Lmo1081)	decreased adhesion to abiotic surface	wall teichoic acid glycosylation	/	Hsu *et al*. (2020)
RmlT (Lmo1080)	decreased adhesion to abiotic surface	wall teichoic acid glycosylation	/	Hsu *et al*. (2020)
BapL (Lmo0435)	decreased adhesion to abiotic surface	LPXTG protein in cell envelope involved in colonization	Lin0457	Jordan *et al*. (2008)
InlL (Lmo2026)	decreased adhesion to abiotic surface	LPXTG protein involved in colonization	Lin0740	Popowska *et al*. (2017)
InlA (Lmo0433)[a]	decreased adhesion to abiotic surface	LPXTG protein virulence factor	/	Chen *et al*. (2008) Travier *et al*. (2013)
InlB (Lmo0434)	decreased adhesion to abiotic surface	GW protein virulence factor	/	Chen *et al*. (2008)
ActA (Lmo0204)	ActA-ActA interaction between cells, enhancing aggregation	cell envelope protein with hydrophobic tail virulence factor	/	Travier *et al*. (2013)
FliP (Lmo0676)	unknown	flagellar biosynthesis	Lin0684	Chang *et al*. (2012) Ouyang *et al*. (2012)
Lmo0707	unknown	flagellar hook	Lin0715	Ouyang *et al*. (2012) Alonso *et al*. (2014)
FlaA (Lmo0690)[a]	unknown	flagellar protein	Lin0698	Chang *et al*. (2012) Alonso *et al*. (2014) Kumar *et al*. (2009) Piercey *et al*. (2016)

The surfactome components presented in the table complement understanding of the schematic representation of *L. monocytogenes* surfactome by more detailed description of proteins, their role in biofilm development and presence of homologous genes in *L. innocua*.
a. Conflicting results among studies and the biofilm phenotype may be either enhanced or decreased.

regulator (*agr*) locus coding for peptide sensing (Zetzmann *et al*., 2019). The Δ*agrD* mutant that does not produce the autoinducing peptide responsible for autoregulation of the *agr* system exhibits decreased ability to form biofilm, and its motility and surface hydrophobicity are not different from wild type. But, its viability is significantly decreased following lysozyme digestion, inferring that *agr* inactivation leads to altered composition of the surfactome resulting in decreased adhesion to the abiotic surface or neighbouring bacteria (Zetzmann *et al*., 2019). The *agr* system is involved also in *S. aureus* biofilm formation, but its inactivation leads to enhanced biofilm phenotype possibly being consequence of decreased accumulation of extracellular proteases and increased expression of surface proteins such as fibronectin binding protein or protein A (Novick *et al*., 1993; Solano *et al*., 2014).

Peptidoglycan is decorated by teichoic acids that are divided into wall teichoic acids (WTAs), which are covalently bound to peptidoglycan, and LTAs, which are anchored to the cytoplasmic membrane by a glycolipid (Fig 1C and E). Because the teichoic acids are charged, they are considered to play an important role in initial adhesion to abiotic surfaces (Bos *et al*., 1999). Though transposon mutant library screens indicated importance of LTA and WTA in biofilm formation process, limited validation studies have been performed in *Listeria* to confirm these findings. LTA structure seems to be conserved among strains and consists of a polyglycerol phosphate backbone substituted by D-alanine and

galactose in *L. monocytogenes* (Nichterlein *et al*., 1997). *L. monocytogenes* synthesizes the LTA backbone by the enzymes LtaP (Lmo0644), a primase that produces glycerolphosphate glycolipids, and LtaS (Lmo0927), an LTA synthase that produces polyglycerolphosphate chains (Webb *et al*., 2009) (Fig 1C). Elongation of the LTA backbone is initiated on a glycolipid anchor synthesized by LafA (Lmo2555) and LafB (Lmo2554) (Fig 1C); mutants lacking these two enzymes produce only minute amounts of LTA (Webb *et al*., 2009). Transposon mutant library screens indicated that *lmo0644* (*ltaP*) and *lmo2554* (*lafB*) mutants exhibit deficient biofilm phenotype on background strains *L. monocytogenes* serotypes 4b and 1/2a at 32, 35 and 37 °C though these findings remain to be validated (Table 1) (Chang *et al*., 2012; Ouyang *et al*., 2012; Alonso *et al*., 2014). *Enterococcus faecalis* LTAs glycolipid anchor is also synthesized by orthologous two enzyme system similarly to *Listeria* and is named BgsA. *Δbgs* mutant contains longer LTA chains with less hydrophobic cells than the wild type and does not differ from the wild type regarding adherence, but differs significantly regarding biomass accumulation over prolonged incubation times, leading to negative biofilm phenotype (Theilacker *et al*., 2009). LTAs in *L. monocytogenes* are substituted by D-alanine (Fig 1 D), and mutants lacking alanine esters exhibit increased electronegativity and more efficient binding of cationic compounds (e.g. colistin, nisin and polymyxin B) (Abachin *et al*., 2002). D-alanylation of LTAs is carried out by the *dlt* operon in gram-positive bacteria, and its activity determines biofilm formation ability in *Staphylococcus aureus* where its inactivation leads to biofilm inhibition on polystyrene and glass due to increased negative charge (Gross *et al*., 2001). Transposon mutants with interrupted *dltB* exhibit decreased ability to form biofilms on abiotic surfaces at different temperatures also on *Listeria* background strains with 1/2a serotype (Alonso *et al*., 2014; Piercey *et al*., 2016). *Listeria* changes LTA composition also in response to temperature changes, rendering LTAs less immunostimulatory and more hydrophobic (Dehus *et al*., 2011). This could lead to differences in biofilm formation ability at different temperatures, but biofilm phenotypes of LTA synthesis associated transposon mutants remained impaired between 15 and 37 °C (Chang *et al*., 2012; Alonso *et al*., 2014; Piercey *et al*., 2016). Homologues of LTA synthesis genes involved in *L. monocytogenes* biofilm formation were also found in *L. innocua* CLIP 11262 (S1 Table); however, their role in biofilm formation is unknown. Furthermore, the structure of *L. innocua* LTAs has not yet been determined. Nevertheless, they exhibit similar potential to stimulate phagocytes to produce proinflammatory cytokines, implying large structural similarities with *L. monocytogenes* LTAs (Nichterlein *et al*., 1997).

Unlike LTAs, WTAs are more diverse and represent *O*-antigens, which are the main determinants of serotypes along with flagellar proteins (H antigens). WTAs are composed of a variety of mono- and oligosaccharides that substitute hydroxyl groups on the 20 to 40 repeating ribitol-phosphate units and are classified into type I and type II (Shen *et al*., 2017). WTA backbone synthesis is not well described in *Listeria* (Fig 1E); however, it has been established that the genes *tagO1* (*lmo0959*) and *tagO2* (*lmo2519*) are required in the initial steps of WTA synthesis and that only double mutants are devoid of WTA (Eugster and Loessner, 2012). The WTA backbone is further substituted by sugar moieties in *Listeria*, and these glycosylation patterns were found to be congruent with the current serotyping scheme (Shen *et al*., 2017). *Listeria* WTA type I are substituted by rhamnose and/or N-acetylglucosamine (Fig 1F and G), and serotypes belonging to this group (1/2, 3, 7) differ only in small mutations in genes coding for rhamnosylation and/or GlcNAcylation causing serotype switch (Eugster *et al*., 2015). Absence of rhamnosylation caused by mutations in genes *lmo1081* (*rmlA*) and *lmo1080* (*rmlT*) in background strain EGD-e (serotype 1/2a) was proven to have negative effect on adhesion and consequently biofilm formation on abiotic surface (Hsu *et al*., 2020). Serotype 1/2a decorates its LTA by galactose and WTAs by N-acetylglucosamine using the same flippase GtcA in both pathways (Rismondo *et al*., 2020). GtcA in strain EGD, serotype 1/2a, is coded by *lmo2549* and works together with the gene *lmo2550* to decorate WTA with N-acetylglucosamine (Fig 1G) (Eugster *et al*., 2011). Mutants Δ*lmo2549* and Δ*lmo2550* constructed by targeted mutagenesis had a more hydrophilic surface compared with wild type and adhered less to stainless steel at 30 °C; biofilm was growing in the form of individual bacterial clusters, and the mature biofilm exhibited architecture that was not carpet-like in comparison with wild-type EGD-e biofilm (Brauge *et al*., 2018). The adhesion was similarly impaired in the strain carrying natural mutation in *lmo2550*, but there were no differences in morphology in mature biofilm compared with wildtype (Brauge *et al*., 2018).

Wall teichoic acids in the serotype 4b belong to type II WTAs where N-acetylglucosamine is incorporated into the backbone and is decorated mostly by galactose or/and glucose, but the effect of glycosylation on biofilm remains unknown in this serotype (Sumrall *et al*., 2020a).

Wall teichoic acids were also found to be the only extracellular carbohydrates in biofilms formed by several different strains belonging to the serotypes 1/2a and 4b (Brauge *et al*., 2016). The structure of extracellular teichoic acid was the same as that of the WTAs found in the cell wall of the biofilm-forming strain. The origin of

WTAs in the extracellular matrix remains unknown; however, the origin is postulated to result from cell lysis or even active WTA export (Brauge *et al*., 2016).

Listeria innocua strains have been traditionally typed as serotype 6, and we can infer that their WTA structure is similar to serotypes 4a and 6b. An exception is *L. innocua* strains that exhibit serotype 4b antigens, because they harbour *gtcA* homologues; however, how this relates to their pathogenic potential and biofilm formation is unknown (Lan *et al*., 2000).

Cell envelope proteins

The *L. monocytogenes* EGD-e genome harbours 133 putative surface proteins out of the total 2853 predicted proteins (Bierne and Cossart, 2007). These putative surface proteins were predicted based on the presence of specific domains or motifs that are known to mediate translocation across membranes and attachment to the cell envelope. They have been classified as follows: (i) proteins covalently attached to peptidoglycan moieties (LPXTG and NXXTX proteins), (ii) proteins non-covalently associated with the surface (proteins with the GW, LysM or Wxl domain), and (iii) membrane-bound proteins (proteins with hydrophobic tails and lipoproteins) (Cabanes *et al*., 2002). Surface proteins, including flagellar proteins, also determine the differences between the EGD (serotype 1/2a, CC9) and F2365 (serotype 4b) strains and may also account for differences in strains' antigenicity and pathogenicity (Donaldson *et al*., 2009). The global proteome of these strains encompasses 1754 proteins (61.6% of the predicted proteome) for EGD and 1427 proteins (50.5% of the predicted proteome) for F2365. According to the function of the identified proteins, these strains were found to perform basic cellular processes similarly. Both strains also express the virulence-related LPXTG protein internalin A and another uncharacterized internalin, whereas F2365 does not express internalin B (*inlB*) because its gene is truncated. However, five LPXTG proteins with unknown function at 37 °C were significantly more expressed in EGD cells, whereas a cell wall anchor protein was significantly more expressed in F2365 cells (Donaldson *et al*., 2009). Comparison of surface proteomes during biofilm growth mode is rarely performed because of the complexity of the extraction and analysis set-up. The highly adherent strain 99-38 from lineage I expresses 21 proteins exclusively during biofilm growth mode and not during growth in planktonic culture, including the surface proteins Lmo0275 (DNA uptake-related protein), Lmo0394 (P60-like protein), Lmo0204 (ActA), Lmo0434 (InlB) and Lmo2713 (internalin-like protein) (Tiong *et al*., 2016). This study also identified moonlighting proteins, including glyceraldehyde-3-phosphate dehydrogenase (Lmo2459) with a yet unknown role at the surface (Tiong *et al*., 2016). Differences regarding surface proteomes have been found among studies that have often been attributed to different experimental temperatures (Calvo *et al*., 2005; Tiong *et al*., 2016). However, a comparative study of *L. monocytogenes* EGD-e grown on stainless steel at 10, 25 or 37 °C has demonstrated that it is highly unlikely that a set of surface proteins could play a unique role in the adaptation to different temperatures, with the exception of motility-related proteins (Santos *et al*., 2019).

The *L. innocua* CLIP 11262 genome also harbours several surface proteins, but not those associated with virulence in *L. monocytogenes* (e.g. InlA, InlB, Ami) (Glaser *et al*., 2001). Surfaceomes (i.e. all surface proteins) of *L. monocytogenes* EGD-e and *L. innocua* CLIP11262 planktonic cultures grown at 37 °C revealed 19 and 11 proteins respectively (Calvo *et al*., 2005). The most abundant species were LPXTG proteins, followed by proteins involved in peptidoglycan synthesis (PBSs, P60 and P45-like proteins) and NXXTG proteins (Calvo *et al*., 2005). In both species, orthologous genes were identified for proteins P60, P45 and NXXTG; however, several LPXTG proteins were absent in *L. innocua,* and some orthologous proteins could not be identified (Calvo *et al*., 2005).

Cell envelope proteins associated with L. monocytogenes *pathogenicity*

The most characterized virulence cell wall protein is internalin A (InlA) because it is crucial for the internalization of *L. monocytogenes* into epithelial cells (Lecuit *et al*., 1997). It belongs to the LPXTG protein group and has a sorting signal at the C-terminal that is postulated to direct protein attachment to peptidoglycan by sortase SrtA, a membrane-bound transpeptidase (Fig 1H). This type of proteins is also the most abundant among predicted cell wall proteins in *Listeria*. Inactivation of the *inlA* and *inlB* genes in *L. monocytogenes* EGD-e was shown to decrease the strain's ability to adhere to glass at 37 °C, and this effect was even stronger in the double mutant (Table 1) (Chen *et al*., 2008). Expression of full-length InlA and InlB exhibited a positive correlation with the ability of *L. monocytogenes* to adhere to glass surfaces at 30 °C; this ability did not depend on the serotype (Chen *et al*., 2009). Unlike serotype 4b, many serotype 1/2c and 1/2a strains from lineage II express truncated versions of InlA (Franciosal *et al*., 2009). This truncated version lacks the cell wall-binding domain and is secreted into the extracellular space where it undergoes proteolytic processing. The resulting peptides seem to enhance biofilm formation as they can be easily incorporated into the biofilm as extracellular matrix

(Franciosal *et al*., 2009). On the other hand, transposon mutants with inactivated *inlA*, *inlB* and *inlH* led to an enhanced biofilm phenotype at 15 °C, but *inlA* and *inlB* are not expressed at lower temperatures, because their transcriptional activator PrfA is expressed only at temperatures above 30 °C (McGann *et al*., 2007; Piercey *et al*., 2016). Yet, Travier et al (2013) found that *ΔinlA* biofilm phenotype was enhanced compared to wild type and *ΔinlB* showed no change in biofilm formation at 37 °C on polyvinyl chloride. Conflicting observations could not be due to serotype related differences, because these studies were done on background strain serotype 1/2a, but could be associated with experimental set-up or a yet unknown aspect of *inlA* and *inlB* expression regulation.

Another member of the internalin family with LPXTG motif, InlL, is also important for adhesion to polystyrene at 30 °C and as it binds secreted mucin type it could have also role in pathogenesis (Popowska *et al*., 2017). InlL is mainly present only in *L. monocytogenes* food isolates (CC9 and CC121), and hence, it might play an important role in the persistence of *L. monocytogenes* (Popowska *et al*., 2017). Another non-core persistence-associated gene with LPXTG motif, *bapL* (*lmo0435*), affects the adhesion of *L. monocytogenes* EGD-e onto polystyrene and stainless steel at 37 °C (Jordan *et al*., 2008). Additionally, strains naturally lacking the *bapL* gene exhibit both stronger and weaker adhesion, suggesting that BapL is not the only determinant of adhesion (Jordan *et al*., 2008). These results also demonstrate that molecular determinants of biofilm phenotype can be isolate or serotype specific and thus results cannot be simply extrapolated to other strains.

InlB is an important virulence factor and crucial for invasion of non-phagocytic human cells and plays a role in adhesion also to abiotic surfaces (Parida *et al*., 1998; Chen *et al*., 2008). InlB is associated with the cell wall by a GW motif-containing domain to WTA via galactose (serotype 4b) or rhamnose (serotype 1/2) and in position-dependent manner and is functional only when glycosylation pattern is complete (Carvalho *et al*., 2018; Sumrall *et al*., 2020a). This implies that glycosylation is responsible for increased virulence via InlB, and its mere expression does not alter strains pathogenic potential; that has been partially confirmed by expression of 4b serotype glycosylation gene *gttA* in serotype 4d causing increased invasiveness of this strain (Sumrall *et al*., 2020b). Even though this was a laboratory validation study, lateral transfer of glycosylation genes happens also naturally among *Listeria* species as demonstrated by *L. innocua* harbouring *gtcA* gene from *L. monocytogenes* leading to altered glycosylation in *L. innocua* (Lan *et al*., 2000). These events imply that surfactome is ever evolving property of the strain resulting in altered phenotype being more or less persistent or invasive.

An important part of *Listeria* surfaceome is autolysins (Fig. 1B), and some are involved in pathogenesis like Ami, and this group of proteins has been described in a previous section due to their role in peptidoglycan turnover.

Membrane protein ActA, responsible for actin polymerization promoting *L. monocytogenes* intracellular motility and cell-to-cell spread, is critical also for biofilm formation (Fig 1) (Travier *et al*., 2013). This protein mediates direct contact between bacteria by ActA-ActA interaction resulting in aggregation, which is very important for biofilm formation. Active ActA enabled formation of larger clusters of bacteria thus promoting thicker biofilm biomass development on glass surface (Travier *et al*., 2013). Furthermore, the expression of *L. monocytogenes* ActA in *L. innocua* increased its ability to form biofilm thus confirming its direct role in biofilm formation (Travier *et al*., 2013).

The flagellar apparatus and motility

Listeria monocytogenes and *L. innocua* can swim in the extracellular environment by using four to six peritrichous flagella, and their motility is temperature-dependent and to a lesser degree also strain-dependent (Peel *et al*., 1988; Gründling *et al*., 2004). Motility is potent at temperatures below 30 °C because the main flagellin FlaA (Lmo0690) is highly expressed. FlaA expression decreases at physiological temperatures of 37 °C, and a small proportion of bacterial populations of certain strains may exceptionally still be flagellated at elevated temperatures (Way *et al*., 2004). In *L. monocytogenes* EGD-e, flagellin synthesis is regulated by MogR (Lmo0674) and independently of PrfA (Lmo0200), a virulence transcription factor that is absent in *L. innocua* (Gründling *et al*., 2004). Flagella proteins of the *L. monocytogenes* serotypes 4b, 1/2c, 1/2a and 1/2b are additionally uniquely modified with O-linked β-*N*-acetylglucosamine at up to six sites on each protein monomer (Schirm *et al*., 2004). All four genome-wide studies identified at least one component of the flagellar apparatus (Fig 1I) that affects the ability of *L. monocytogenes* to form biofilms (Chang *et al*., 2012; Ouyang *et al*., 2012; Alonso *et al*., 2014; Piercey *et al*., 2016); however, their results are conflicting. Although the natural variability in the motility of *L. monocytogenes* is large, this property was only found to correlate with the ability to form biofilms on stainless steel where motile strains formed weaker biofilms (Di Bonaventura *et al*., 2008). *L. monocytogenes* 568 (serotype 1/2a) transposon mutant with interrupted *lmo0690* (*flaA*) was able to form biofilms on polystyrene or stainless-steel surfaces at 15 °C but not on polystyrene peg lids (Piercey *et al*., 2016). A FlaA transposon mutant on background strain 10403S (serotype 1/2a, CC7)

exhibited a decreased ability to form biofilms on polystyrene at 35 °C (Alonso *et al*., 2014). This finding was confirmed by targeted mutagenesis in strain EGD-e (serotype 1/2b, CC9) where Δ*flaA* formed less dense biofilm compared with the wild type at 30 °C detected by crystal violet staining and electron microscopy (Kumar *et al*., 2009). But, in both studies, biofilm screening temperatures were above 30 °C when motility decreases in *Listeria*, whereas at 15 °C, it is fully expressed, and we can hypothesize that biofilm did not form on the peg lids, because motility is required to swim towards the peg, whereas it is not required to form biofilm on the microplate bottom. Regulation of flagellin gene expression is extremely complex and may be affecting biofilm phenotypes by a yet unknown mechanism that would explain inhibited biofilm formation of Δ*flaA* at higher temperatures (Cossart, 2011). Discrepancies between studies could also be due to the variability of biofilm assays and sample preparations. Biofilm staining techniques mainly use rigorous washing of biofilms, leading to the detachment of weakly bound bacteria, resulting in possible falsely negative biofilm phenotype.

Antimicrobials targeting the surfactome

Understanding of *L. monocytogenes* surfactome components and associations between them is still limited, and only limited antimicrobials target them. The treatment of choice against listeriosis is the use of ampicillin or gentamicin belonging to the beta lactam group of antibiotics (Olaimat *et al*., 2018). They target the PBPs described above in the section 'Cell envelope carbohydrates' and play key roles in peptidoglycan biosynthesis. Penicillins usually work bacteriostatically and where shown to inhibit growth of planctonic bacteria and biofilm formation close to minimal inhibitory concentration (MIC), but enhanced biofilm formation at much lower concentrations (Nguyen *et al*., 2012). Several beta lactams were shown also to disperse mature biofilm at concentrations above MIC, but a small proportion of bacterial population survived treatment regardless of increasing antibiotic dose implying dispersal needs peptidoglycan turnover, which is absent in dormant bacteria (Nguyen *et al*., 2012).

Among alternative approaches, sortases have been suggested as antimicrobial targets for many gram-positive pathogens because they are responsible for the anchoring of LPXTG proteins, many of which are virulence factors. The majority of LPXTG proteins in *L. monocytogenes* are substrates of sortase A (SrtA) (Bierne *et al*., 2002). The flavonoid chalcone, used in traditional Chinese medicine, was found to inhibit SrtA with an IC50 of 28.41 $\pm$ 5.34 μM by binding to the same active region as the substrate LPTTG (Li *et al*., 2016). At effective concentrations, chalcone reduces *L. monocytogenes* invasion of Caco-2 cells (Li *et al*., 2016). Another chalcone compound, phloretin, which is naturally present in apples, was also shown to inhibit SrtA in *in vitro* assays (Wang *et al*., 2017). *L. monocytogenes* EGD-e pre-treated with phloretin exhibits a diminished ability to invade Caco-2 cells, and the addition of phloretin after invasion reduces the multiplication of intracellular bacteria. Similar effects were also observed using baicalein, a flavonoid from *Scutellaria baicalensis* (Lu *et al*., 2019). The effect of these compounds on biofilm formation has not been evaluated to date.

Furthermore, bacteriophages also exploit surface structures as receptors and have gained a lot of interest as alternative antimicrobials. Phage P100 is commercially available in the United States for use against *L. monocytogenes*, *L. innocua*, and other *Listeria* spp. on foodstuff and has a status of 'generally recognized as safe' (GRAS). WTA glycosylation is directly affected by bacteriophage predation causing switches between *L. monocytogenes* serotypes with WTA type I (Eugster *et al*., 2015). Resistance mechanism against bacteriophages in serotype 4b was also found to be associated with altered WTA and LTA structures, which also abolish its ability to invade Caco-2 and HepG2 cell lines (Sumrall *et al*., 2019). Altered glycosylation evidently affects bacterial surfactome and consequently interactions with environment as demonstrated by 4b serotype bacteriophage resistance and 1/2a serotype biofilm phenotypes, but it remains to be elucidated if and how it affects strain's persistence.

Concluding remarks

Understanding the correlation between pathogenicity potential and key surface structures important for biofilm formation in *L. monocytogenes*, which enables long-term persistence, will facilitate informed targeting of the strains that pose the highest risk to human and animal health. Insight into the molecular make-up of bacterial surfaces can provide new ideas and directives for the development of novel antimicrobial strategies to prevent the development of bacterial resistance and extend our antimicrobial armoury.

Cell envelope carbohydrates present an important source of novel antimicrobial targets that are expected to expand as a result of the growing availability of glycobiology tools. They do not affect just overall properties of the surface like charge and hydrophobicity, but also direct localization of proteins composing the surface proteome. The latter has been characterized mainly in terms of pathogenic potential of *Listeria*, but as shown, these proteins play roles also in biofilm and possibly persistence. According to currently available knowledge, surfactome components contribute to adhesion to abiotic

surfaces as expected, with exception of ActA mediating cohesion, the cell-to-cell interactions thus promoting aggregation and biofilm development. The role of LTA and some other components remains to be validated. Surfactome properties are further differentiated among serotypes, phylogenetic groups or clonal complexes as demonstrated by differences in WTA decoration and presence of LPXTG proteins.

Surfactome has been modulated using natural antimicrobials via sortase inhibition or bacteriophage treatment resulting in altered behaviour in cell line invasion models. Similar surfactome remodelling could lead to inhibition of biofilm process thereby negatively affecting the *Listeria* persistence. Based on the current research, the surface components of *L. monocytogenes* and *L. innocua* that promote biofilm formation differ and may thus cause differences in the responses to antimicrobials that target specific surface structures, but the classes of surfactome components are expected to be related to certain *L. monocytogenes* serotypes.

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Conflict of interest

The authors declare that they have no competing interests.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. List of proteins affecting *Listeria monocytogenes* biofilm formation on abiotic surfaces.

Appendix S1. Interaction network analysis procedure and results.

