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# Recent advances on the interaction of glycolipid and lipopeptide biosurfactants with model and biological membranes



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### Abstract

Microbial biosurfactants have gained interest in the last decades because of their unique characteristics. The variety of chemical structures within these compounds makes them very versatile, with glycolipids and lipopeptides outstanding among the rest. The amphiphilic nature of these compounds makes them to partition into and strongly interact with phospholipid membranes, modifying their structure and function. Thus, much research has been done on the characterization of the interaction of alvcolipid and lipopeptide biosurfactants with model and biological membranes. Whereas the studies involving phospholipid model membranes were mostly carried out earlier, most of the recent research has focused on biological membranes, including mammalian and microorganisms' systems. This review presents the recent developments achieved on the interaction of the main glycolipid and lipopeptide biosurfactants with model and biological membranes.

#### Addresses

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### Keywords

Biosurfactant, Glycolipid, Lipopeptide, Phospholipid membranes.

### Introduction

Among microbial biosurfactants, glycolipids and lipopeptides stand out for their unique physicochemical properties and extraordinary biological activities. Certainly, these two groups of biosurfactants are by far the most widely used in all kinds of industrial applications, some of their members, namely rhamnolipids and surfactins, being among the most cited in the scientific literature within this field. The marked amphiphilic nature of biosurfactants makes them very prompt to readily partitioning into phospholipid membranes and, consequently, biological membranes constitute one of the principal sites where biosurfactants exert their outstanding activities. Since the early works in the 1980's, many studies have been devoted to the investigation on the interaction of biosurfactants with model phospholipid membranes and biological membranes. This article reviews recent experimental studies on the interaction of the main classes of glycolipid and lipopeptide biosurfactants with phospholipid and biological membranes, emphasizing on molecular aspects.

# Interaction of glycolipid biosurfactants with membranes

The amphiphilic and surface-active properties of glycolipid biosurfactants allow them to interact with cellular membranes, and in this way, they may modify the membrane structure and therefore affect the function of the cell. There is an increasing body of evidence showing the potential biological functions of glycolipid biosurfactants, including antibacterial, antiviral and anticancer activities [1,2]. However, the molecular mechanisms underlying these activities are not sufficiently known, and therefore a comprehensive study of the interaction between glycolipid biosurfactants and membranes is required. Next, we will review the studies on the interaction between glycolipid biosurfactants and membranes, which have been recently carried out to get insight into the molecular mechanism of these biosurfactant-membrane interactions.

### Physical properties of the membrane

Biomimetic membrane model systems offer an alternative platform to natural membranes, and allow investigation of lipid/phospholipid and protein/phospholipid interactions under controlled and well defined conditions [3]. The basic structure of any membrane is the lipid bilayer, and different models have been developed to mimic the fundamental structural and functional properties of this bilayer. Some of these models include Langmuir monolayers, lipid vesicles or liposomes, supported solid bilayers, etc. Each one of these systems has its own advantages or disadvantages but, in any case, all of them have been deeply analyzed and characterized using different physical techniques. These studies have shown that model systems can simulate in a very precise way the specific characteristics of a membrane, opening the possibility of systematically investigating membranerelated processes [4]. One of the great advantages of these systems is the possibility of modulating bilayer composition, using both synthetic and natural lipids [5].

The specific lipid composition of the membrane can modulate its physical properties and these physical properties can regulate cellular processes. The main physical properties of membranes include the charge, and the size and precise conformation of the polar group, which together with its local density can directly influence lipid/lipid and protein/lipid interactions. For instance, many examples are known of how polar groups determine the interaction of peripheral proteins with the membrane [6]. The fluidity or rigidity of the membrane is another important physical characteristic. Movements within the membrane are complex and highly anisotropic since they vary considerably from the membrane-water interface, where there is a relatively high degree of order, to the center of the bilayer where disorder is much greater [7]. This property has a very important effect on the freedom with which molecules move and interact with each other [8]. Long saturated acvl chains in lipids lead to increased order, while increased presence of unsaturations and short acyl chains decrease lipid order in the membrane [8].

The formation of membrane domains is an aspect that has received significant attention in recent years. It is generally accepted that biological membranes are not uniform, but that there are regions in which certain components are concentrated. One class of these domains are the regions enriched in cholesterol and sphingomyelin (SM) that are called lipid rafts [9]. These domains can be easily detected in model membranes where they have a size of dimensions of microns [10].

Table 1

The thickness of the membrane is another very important property that depends on the lipid packing, the length of the acyl chains and the presence of other components such as cholesterol [11]. It has been suggested that membrane thickness has a large impact on the sorting and organization of proteins in cell membranes, because a so-called hydrophobic mismatch can occur between the length of the transmembrane domains of proteins and the thickness of the membranes [12].

In the membrane, lipids are held in place due to the hydrophobic effect. This is the way that hydrophobic acyl chains stay away from water. However, this situation is not entirely satisfactory for lipid molecules, since, being confined in a bilayer structure together with their neighboring molecules, they are subjected to great stresses. These various stresses and interactions cause a heterogeneous distribution of pressure across the membrane, giving rise to one of the most interesting and fundamental properties of the membrane: the lateral pressure profile [13].

Thus, biomimetic membrane model systems have proven to be a valuable tool to the study of the interaction of biosurfactants with membranes, as shown by the vast number of publications reviewed below (see Table 1 for a summary).

### Rhamnolipids

The predominant classes of rhamnolipids (RL) are monorhamnolipids (mono-RL) and dirhamnolipids (di-RL), these biosurfactants are made of one or two rhamnoses respectively, linked to a hydroxy fatty acid (Figure 1). Probably RL are the most investigated glycolipid biosurfactants up to now, due to their notable characteristics and variety of utilizations [14]. RL biosurfactants have gained interest in the biomedical field due to its antimicrobial activities [15].

Glycolipid biosurfactants	System	Effects	Reference
Rhamnolipids			
	PLPC	Less hydrogen-bonded state of carbonyl and phosphate groups.	[28]
	POPC/PLPC	Location near the lipid phosphate group close to the phospholipid glycerol backbone.	[28]
	PLPC/POPC $-^{2}H_{31}$ ; soy PC/POPE $-^{2}H_{31}$ / soy Pl/soy PG/ $\beta$ -sitosterol/soy GCer (plant plasma model membrane)	No disturbance of the dynamic of the phospholipids.	[28]
	POPC- <sup>2</sup> H <sub>31</sub> /POPG/ergosterol (fungi model membrane)	Increase in the dynamic of the membrane hydrophobic core.	[28]
	DMPS	Widening and shifting to lower temperatures of the gel to liquid crystalline phase transition, showing a gel-phase immiscibility. Decrease of the order of the acyl chains and increment of the carbonyl groups hydration.	[37]

Table 1 (continued)

Glycolipid biosurfactants	System	Effects	Reference
		Location at the center of each monolayer	
	DPPC	close to the carbonyl groups. Widening and shifting to lower temperatures of	[38,39]
		the gel to liquid crystalline phase transition.	[00,00]
	DOPE/DPPG (bacterial model membrane)	No significant differences in Laurdan	[38,39]
	DPPC/DPPG/DOPC/DOPG/cholesterol	Widening and shifting to higher temperatures	[38,39]
	(raft like model membrane)	of the gel to liquid crystalline phase transition, and an increment of the general lipid order parameter in the liquid crystalline phase. Increase of the bilayer thickness difference between the two liquid phases	
		Partition into all-fluid one-component lipid bilayers, causing leakage and changing size	
	HSPC/DSPE-PEG/cholesterol	Morphological changes in liposome laver.	[40]
	POPC; DOPC/SM/cholesterol (raft like model membrane)	Alteration of membrane spontaneous curvature and liquid ordered domain protrusion	[41]
	DPPC	Release of redox probe.	[42]
	DPPC/GM1	Affinity of RL to the membrane was promoted	[43]
	DOPC/SM/cholesterol	Interaction between RL and vesicle occurs by insertion and pore formation.	[44]
	DOPC/sterols	The interaction of RL with bilayers depends on the procedure of liposome preparation and the presence of different sterols.	[45]
	POPC/POPE/POPS/PIP1/ergosterol; DOPA/DOPC/POPE/DOPE/POPI/POPS/ DOPS/ergosterol	RLs were inserted below the phospholipid phosphate group, forming ionic bonds between the carboxylate group of RL and the amino group of the phospholipids headgroups, and forming stable adducts with eroosterol.	[46]
Trehalose lipids			
	Complex lipid composition modeling normal and cancer cell membranes	The selectivity of TL for cancer cells is caused by the difference of the lipid compositions in the extracellular side of the cellular membrane	[49]
	PA/PE	TL affects fusion by increasing the hydration force of the membrane or acting as a steric barrier.	[52]
Mannosyl erythritol lipids			
	DPPC	Pretransition disappeared and the main gel to	[59]
	EYL	MEL-A caused changes in lipid curvature associated with its partitioning into the layer of phospholipids.	[60]
Sophorolipids			
	DOPS/DOPC/DSPE-PEG-Biotin/DSPE- ATTO655	Acidic pH and charges are dominant features underlying the interaction of SL with membranes.	[65]
	DPPC	SL produced fluid phase immiscibility. SL increased ordering of the phospholipid acyl chain palisade and hydration of the lipid/water interface.	[66]
	POPC	SL induced membrane permeabilization could be due to the formation of laterally segregated domains.	[66]

RL have shown excellent antibacterial activities, and though their exact antibacterial mechanism of action is not properly known, several reports pointed that their interaction with the cell membrane would be critical. In recent years there has been great interest in studying the interaction between RL and bacterial membranes.





The chemical structures of the four glycolipid biosurfactants reviewed.

With this background, Sana et al. [16] studied the bacterial cell membrane disrupting ability of RL synthetized by Pseudomonas aeruginosa C2 against Staphylococcus aureus ATCC 25923 and Escherichia coli K8813. The authors treated overnight grown bacterial cultures with the RL and assessed the effect of the biosurfactant on the bacterial membrane through different experimental approaches. The bacterial membrane permeabilization was evidenced by the increase in protein content release together with the change of cell surface hydrophobicity towards n-hexadecane. Significant membrane permeability against both bacteria was detected also by the increase of crystal violet up taking efficiency, and the increase in the presence of UV-absorbing materials released from the cells after the treatment was taken as an indication of the cell disintegration. External deformities in cell surface as seen by scanning electron microscopy (SEM) micrographs were taken as a proof of the cell membrane disrupting capabilities of the RL. The rough surfaces found by the authors indicated the deformation of the cells due to membrane disruption and pore formation. This methodology has been broadly applied in most of the studies investigating the effects of biosurfactants on diverse biological membranes. Interestingly, the author found that all the above-mentioned membrane perturbations were synergistically enhanced when the RL was used in combination with a lipopeptide

biosurfactant and this opened promising alternatives for antibacterial treatments.

RL have been shown to induce specific modifications to the *S. aureus* membrane, they decreased surface charge, stimulated a lasting increase in membrane fluidity as seen by Laurdan generalized fluorescence, and induced small-molecule permeability observed by enhanced leakage of intracellular ATP into the medium [17]. Radlinski et al. concluded that this alteration of the bacterial membrane was responsible for the capacity of RL to stimulate proton motive force-independent aminoglycoside uptake which led to a restoration of the sensitivity to otherwise tolerant populations of *S. aureus*.

The antibacterial activity of RL against Gram-positive bacteria have been shown to be pH dependent and favored at more acidic conditions by de Freitas et al. [18] who showed that the sensitivity to RL was associated with reduction on cell surface hydrophobicity and cytoplasmic membrane damage. These authors evidenced possible alterations in the chemical composition of the bacterial surface promoted by RL by using Fourier transform infrared (FTIR) spectroscopy. According to the authors, the reduction in intensities of transmittance bands detected for functional groups was originating from the release or remove of cell components by RL,

whereas the increase in vibration intensity of another distinctive functional groups was probably a consequence of the disruption effect of RL exposing the molecules present at inner parts of cell wall/membrane. Similar to that found previously by Sana et al. on S. aureus membranes [16], the authors found comparable leakage of UV-absorbing materials and alteration in bacterial cell roughness as seen by SEM after treatment with biosurfactants of Listeria monocytogenes and Bacillus cereus membranes. However these authors found a reduction of the cell surface hydrophobicity which was at difference with the increase of this parameter found previously [16]. SEM analysis has been also used to show *E. coli* cell wall and membrane disintegrations by a novel RL-like biosurfactant from Lactobacillus casei, revealing the role of membranes as main target of RL [19].

The effect of RL on *S. aureus* cell viability and membrane permeability were investigated by Saadati et al. [20] using flow cytometry and two fluorescent dyes, fluorescein diacetate and propidium iodide. The authors found that the more the cell membrane loses its integrity after treatment with RL the more evident the decrease of cell viability.

Allegrone et al. [21] studied the ability of RL to modify *S. aureus* cell surface hydrophobicity and membrane permeability in comparison to that of two commonly used commercial synthetic surfactants, the authors found that natural RL altered membrane properties more efficiently than Tween 80 and Triton X100.

RL have been also reported to display potential antifungal properties. For destructive fungi in the plant, Platel et al. [22] have recently established that RL showed significant antifungal activity against Zymoseptoria tritici, which is currently the most devastating foliar disease on wheat crops worldwide. These authors measured the growth of fungal colonies on solid medium, and they found that RL with 12 carbon fatty acid chain were the most effective regarding direct antifungal activity. Their results suggested that RL interact directly with the fungus cell membranes which leads to a strong membrane destabilization and cell death. Similar studies but using transmission electron microscopy (TEM) were carried out by Rodrigues et al. [23] on Aspergillus flavus, who demonstrated that RL damaged the cell wall and the cytoplasmic membrane of the fungus therefore explaining the growth inhibition observed.

Experiments on *Botrytis cinerea* and *Sclerotinia sclerotiorum* fungi responsible for diseases in numerous plant species worldwide were performed by Botcazon et al. [24]. In addition to SEM images exhibiting loss of mycelial structure, these authors presented permeability observations using fluorescence dyes and confocal fluorescence microscopy which showed how loss of membrane

integrity can lead to changes in the cells such as permeability, cell death mechanism inductions, modification of the mycelium structure and inhibition of the fungal growth.

These sorts of studies have been carried out also on fungi responsible of infections in humans and animal alike. Sen et al. [25] investigated the effect of RL on *Trichophyton rubrum* which is responsible of the most prevalent superficial mycoses worldwide. Observations involving SEM and atomic force microscopy (AFM) revealed severely altered hyphal morphology in the presence of RL, and examination of the membrane disruptive effect measured by using fluorescent dyes and confocal laser scanning microscopy showed a clear loss of cell membrane integrity.

It is known that RL show antiviral activity against various enveloped and non-enveloped viruses probably through the destruction of viral lipid membranes and capsid [26]. Recently Giugliano et al. [27] evidenced that RL were able to show antiviral activity against Herpes simplex virus type 1 and SARSCoV-2, and in both cases the antiviral activity seems to be related to an action directed on the lipid envelope. To demonstrate this hypothesis, the authors visualized the effect of RL treated viral membranes under TEM showing a disruption in the envelope integrity.

In addition to the previously commented direct antimicrobial properties exerted by RL, some studies have reported their potential interest in plant protection by triggering plant defense mechanisms. An example or this kind of study is the work presented by Monnier et al. [28] who investigated the elicitor and protective activities of RL on annual crops of agronomic interest. These authors studied the RL protective effects of Brassica napus foliar tissues toward the fungus B. cinerea. The authors measured the conductivity of foliar disks after RL treatment and observed and increase in membrane electrolyte leakage, and presented scanning confocal microscopic cell deaths observations which indicated that RL triggered a hypersensitive responselike defense in this plant. Recently Schellenberger et al. [29] showed that RL activate an atypical immune response in Arabidopsis thaliana which was affected by the sphingolipid composition of the plasma membrane, and suggested that direct interaction of the biosurfactants altering the order of the plasma membranes were necessary for the plant response.

RL have been established to show potential anticancer effects and a possible mechanism using membrane remodeling has been proposed [2]. Observations using inverted phase-contrast microscopy and fluorescence microscopy revealed the membrane apoptotic characteristics in RL treated MCF-7 human breast cancer cells [30]. Shen et al. measured membrane tension using a microfluidic device and they showed that RL exhibited selective lysis correlated strongly with cortical membrane tension in different types of cells [31]. Human chronic myeloid leukemia K562 cells exhibited greater cortical membrane tension that healthy blood cells and the authors showed that these cancer cells were more sensitive to RL, the latter being important for its potential application of RL for treatment of blood cancer. Recently, Twigg et al. [32] reported that RL were significantly cytotoxic to two cell lines originally isolated from colorectal tumors (HCT-116 and Caco2). The authors performed a study by direct imaging and bright field microscopy and showed that RL treated cancer cells had their membrane disrupted, which was taken as an indication that the surface-active properties of RL induced necrotic pathways of cell deaths.

All the research described above has revealed that most of the beneficial properties and applications of RL are linked to the potential effects of these biosurfactants on the different types of biological membranes. The heterogeneous organization of lipids, together with the wide variety of lipids with different and distinctive physical properties, makes biological membranes extremely complex structures [33]. Furthermore, covalent associations with proteins and carbohydrates further complicate the structure of the membrane.

This great complexity and diversity of biological membranes prevents the study of the molecular interaction between RL with the membrane. To overcome these obstacles, a series of simplified models of the membrane have been developed that focus on lipids, which are the main constituents of membranes. Biomimetic membranes are systems whose organization best mimics the lipid arrangement found in natural membranes. These models offer an alternative way to study the interactions of compounds of biological interest with the membrane and allow specific investigations under highly defined and controlled conditions. These systems are suitable in vitro tools to study the properties of the membrane and its interactions with biosurfactants, these membrane models can mimic the lipid content of biological membranes under both physiological and pathological conditions, and therefore can be used to study several parameters that can predict the interaction of these biosurfactants with the membrane [34].

The interaction of natural RL with membranes mimicking those of plant and fungi considering the number of double bonds, charges, and different species of lipids, was carried out by Monnier et al. [35] employing FTIR spectroscopy and deuterium nuclear magnetic resonance (H<sup>2</sup>-NMR) techniques. Palmitoyllinoleoylphosphatidylcholine (PLPC) was chosen as a simple model of the plant membrane, and the authors found that in the presence of RL the maximum of the infrared absorption bands corresponding to the carbonyl and phosphate groups appeared at higher wavenumbers. which meant a less hydrogen-bonded state of these groups. Their results suggested that the effect of RL was similar for phospholipids of comparable chain length and was not affected by the number of double bonds. The authors performed a molecular dynamics simulation (MD) of a mixed system formed by mono and di-RL with palmitoyloleoylphosphatidylcholine (POPC)/ PLPC and determined a location for RL near the lipid phosphate group close to the phospholipid glycerol backbone. To obtain a biomimetic bilayer more alike to the plant plasma membrane, the authors made more elaborate bilayers and included new phospholipids species like POPC/POPE/soy phosphatidylinositol (PI)/ soy phosphatidylglycerol (PG)/β-sitosterol/soy GCer. The H<sup>2</sup>-NMR spectra of these complex bilayer were very similar both in the absence and presence of RL, implying that the dynamics of lipid was not affected by RL. However, when the complex biomimetic system imitated the fungi membrane and ergosterol was present in the bilayers, they found an apparent decrease of the spectral width. This change in the spectral width was interrelated to an increase in the dynamic of the membrane hydrophobic core and correlated to the definite structure of ergosterol, emphasizing the role of ergosterol in the alteration of membrane properties exerted by RL.

The effects of RL on the structural and dynamics properties of model bilayers formed by the most abundant membrane phospholipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE) have been extensively characterized in the past by using different biophysical techniques [36]. Phosphatidylserine (PS) is the most relevant negatively charged glycerophospholipid in biological membranes, and Oliva et al. [37] have recently carried out the examination of the phase behavior of mixtures of RL with dimyristoylphosphatidylserine (DMPS). As indicated by differential scanning calorimetry (DSC), the presence of RL in the bilayer produced a continuous widening of the gel to liquid crystalline phase transition, with a decrease of the onset transition, correlating with a decrease of the cooperativity of the transition. The system showed a gel-phase immiscibility indicating formation of a complex which separated from the major part of the phospholipid, and melted at lower temperature.

To look into the influence of RL on PS structural properties, the authors used FTIR spectroscopy. The presence of RL produced a progressive displacement of the maximum of the methylene absorption band to higher wavenumbers, indicating that the number of *gauche* rotamers was higher and a decrease of the order of the acyl chains was concluded. Oliva et al. also considered the effect of RL on the interfacial groups of PS bilayers, and observed that they produced an increment the population of hydrated carbonyl groups [37]. When the authors used MD to simulate the RL interaction with the phospholipid bilayer, they found that the biosurfactant was located at the center of each monolayer close to the carbonyl group of the phospholipid allowing the formation of hydrogen bonds with water (Figure 2).

To increase the knowledge on the interaction of RL with membranes, Herzog et al. [38] examined the interaction between a purified mono-RL and biomimetic membranes containing different anionic phospholipid and using different spectroscopy and microscopy techniques. To envision lipid domains, the authors used fluorescence Laurdan spectroscopy, and found that when RL was present in dipalmitoylphosphatidylcholine (DPPC) bilayers the gel to liquid crystalline phase transition was broader and took place at lower temperatures indicating a disorganization of the gel phase. The incorporation of mono-RL into mixtures of dioleoylphosphatidylethanolamine (DOPE) and dipalmitoylphosphatidylglycerol (DPPG) system showed no significant differences in the presence of mono-RL, but when the system was formed by a heterogeneous fivecomponent anionic raft like membrane model (DPPC/ DPPG/dioleoylphosphatidylcholine (DOPC)/DOPG/ cholesterol), the transition between the two-phase region liquid ordered/liquid disordered was slightly widened and appeared at higher temperatures, attributable to important changes in the lateral organization of the phospholipids and an increment of the general lipid order parameter in the liquid crystalline phase.

Herzog et al. [38] also performed AFM measurements to study the interaction between mono-RL and a mixture of five different anionic phospholipids model

Figure 2



A representative snapshot of an equilibrated DMPS bilayer containing di-RL at 318 K from the MD simulations. Water is shown in red and white sticks, DMPS in green lines and di-RL in sticks with carbon chains in orange. Data taken from the study by Oliva et al. [37].

membrane that mimicked the rafts composition. The addition of mono-RL to the system produced an increase of the bilaver thickness difference between the two liquid phases and a reversal of the normal phase proportion. The authors used giant unilamellar vesicles with the five different anionic phospholipids resembling the raft system and marked them with different fluorescence dyes partitioning in different phases to observe morphological changes trough fluorescence microscopy techniques. Their results demonstrated that the incorporated mono-RL did not change the phase lateral organization of phase-separated heterogeneous membranes, but also that the biosurfactant partitioned into all-fluid one-component lipid bilayers and caused leakage and changes in size and morphology of the lipid vesicles over time.

In this way, Herzog et al. [39] continued their study and examined the interaction of purified mono-RL, di-RL and their precursor, the 3-(3-hydroxydecanoyloxy) decanoic acid, with heterogeneous anionic model membranes systems. Using AFM, confocal fluorescence microscopy, DSC, and Laurdan fluorescence spectroscopy, the authors found that the partitioning of the three compounds into phospholipid bilayers changed in an effectively way the phase behavior, fluidity, lateral lipid organization and morphology of the phospholipid membranes depending on the headgroup structure of the RL and affecting its packing and hydrogen bonding capacity.

The interaction between RL and model membranes has been investigated using phospholipid-based liposomes immobilized on a gold substrate by the multiparametric surface plasmon resonance technique (MP-SPR). Belkilani et al. [40] used biotinylated liposomes formed by hydrogenated soybean phosphatidylcholine (HSPC), distearoylphosphatidylethanolamine-N-[biotinyl(polyethylene glycol)-2000] (DSPE-PEG)/ cholesterol, and found that the injection of RL to immobilized liposomes caused definite variations of the surface plasmon resonance signal, showing that RL interacted with the liposome layer causing morphological changes that could be assessed by modeling the resonance signal.

Recently, Come et al. [41] used analysis of optical microscopy data from giant unilamellar vesicles dispersed in aqueous solutions containing a RL mixture. Vesicles were formed by a single lipid POPC and a ternary system containing DOPC/sphingomyelin (SM)/cholesterol, which resembled lipid raft platforms. Their results demonstrated that RL have a low partition in the lipid bilayer in respect to the total molecules in solution. The authors assumed that RL insert in the outer leaflet with low propensity to flip-flop. In the case of POPC vesicles, the insertion of RL molecules in the outer leaflet impaired changes in spontaneous membrane curvature with incubation time. When the model membranes contained a phase coexistence, the interaction was higher for the liquid disordered phase, and that could alter the membrane spontaneous curvature which, coupled to the change in the line tension associated to the domains' boundary, conducted to liquid ordered domain protrusion.

The interaction of RL with DPPC liposomes was detected using the redox liposome single impact electrochemistry (LSIE) by Luy et al. [42]. Liposomes encapsulated potassium ferrocyanide as an electrochemical active probe, and when RL perturbed the phospholipid bilayer the concomitant release of the redox probe produced impacts at a Pt ultramicroelectrode, and current spikes appeared in the chronoamperometry measurement.

The insertion of di-RL in model membranes made of phospholipids (DPPC) and glycosphingolipids (GM1) has been studied by Rondelli et al. [43] using X-ray scattering, neutron reflectometry and molecular dynamic simulations. The authors found that the affinity of di-RL to the membrane was promoted by the presence of the glycosphingolipids and MD revealed that this fact was related to sugar-sugar attractive interactions at the membrane surface.

In an effort to understand the mechanisms by which RL interacts with cell membranes, Marega Motta et al. [44] accomplished phase-contrast and fluorescence microscopy experiments on membrane models represented by giant unilamellar vesicles made of DOPC/SM/cholesterol, which showed liquid order liquid disorder phase coexistence. The authors elaborated a new method to determine area and volume of giant vesicles with asymmetrical shape and estimated a kinetic model which described the interaction between RL and vesicle in terms of two mechanisms, insertion, and pore formation. Marega Motta et al. detected that mono-RL inserted into the lipid bilayer, induced lipid mixing but it did not formed pores, whereas di-RL after insertion formed pores and modified membrane permeability.

Potapov et al. [45] examined the interaction of RL with model membranes made of DOPC, and studied the dependence of this interaction on the procedure of liposome preparation and the presence of different sterols. The authors used <sup>31</sup>P NMR and ATR-FTIR spectroscopies to study the systems. In the case of the liposome preparation by the film method from a mixture of phospholipid and RL, the authors found that the mobility of the head group of the phospholipid increases, the conformational disorder of the hydrophobic tail increases, and the degree of hydration of the carbonyl and phosphate groups of the phospholipid decreases. Potapov et al. assumed that RL were incorporated into the membrane in the form of clusters and were located closer to the middle of the bilayer. In the case of the liposome preparation by injection of RL solution to preformed liposomes, the author found that RL molecules migrated into the membrane in the form of individual molecules and were located closer to the head portion of phospholipids. In addition, the authors found that the sterol composition of the model membrane also affected the interaction of RL with the membrane.

Most recently, Rodríguez-Moraga et al. [46] described the effect of RL on complex model membranes, formed hv POPC/palmitoyloleoylphospatidylethanolamine (POPE)/palmitoyloleoylphosphatidylserine (POPS)/ phosphatidylinositol phosphate (PIP1)/ergosterol, and dioleoylphosphatidic acid (DOPA)/DOPC/POPE/ dioleoylphosphatidylethanolamine (DOPE)/palmitoyloleoylphosphatidylinositol (POPI)/POPS/dioleoylphosphatidylserine (DOPS)/ergosterol, mimicking the membrane of two different fungi by using MD. The authors suggested that RL were inserted below the phospholipid phosphate group, forming ionic bonds between the carboxylate group of RL and the amino group of the phosphatidylethanolamine or phosphatidylserine headgroups. In addition, the authors found that RL formed stable adducts with ergosterol.

# Trehalose lipids

An important group of trehalose lipids (TL) is formed by succinoyl-TL that have been isolated specially in *Rhodococcus* genus (Figure 1). These biosurfactants have the capacity to lower interfacial tension and increase pseudosolubility of hydrophobic compounds, therefore the interest on these compounds have raised due to their promising utilization in a series of fields [47]. In the last decades, a number of authors used biophysical techniques to characterized the interaction between TL and model bilayers made by individual phospholipid species [36].

Recently, Nikolova et al. [48] determined the anticancer activity of TL on breast cancer cell. The authors used cell viability and wound healing assays together with cell morphology imaging assays to observe changes in morphology, adhesion, viability, migration, and the possibility of forming colonies in cancer cell lines induced after treatment with TL. The authors explained their results by suggesting a mechanism by which TL interacted and penetrated the outer leaflet of the membrane, segregated, and provoked the appearance of asymmetry between both monolayers that could result in membrane invagination and endosome formation.

More recently, Hirano et al. [49] used coarse-grained MD to suggest a molecular mechanism to explain the selective attack of TL on cancer cells. The authors inferred that this selectivity was caused by the difference of the lipid compositions in the extracellular side of these cellular membranes: lipids possessing bulky and hydrophilic head groups, such as PI and PS, are abundantly present in the extracellular side of the cancer cell but absent in the case of the normal cell. These phospholipids could cause the cancer cell membrane to bend into a large positive curvature that favored tight contact between the cellular membrane and the TL.

Another important TL is trehalose dimycolate (TDM), which contains a a-branched chain mycolic acid esterified to the C6 position of each glucose. This TL is the basic component of the cell wall glycolipids in *Mycobacteria* and *Corynebacteria*, commonly named cord factor, and comprises an important factor in the virulence of these bacteria [50]. TDM has been extensively studied from a medical point of view due to the fact that it plays a central role in pathogenesis during infection and also showed a number of different biological activities [51].

Using a fluorescence assay, Spargo et al. [52] determined that the presence of TDM was very efficient at inhibiting calcium induced fusion between large unilamellar vesicles made of phosphatidic acid (PA) and PE, suggesting that TDM could affect fusion either increasing the hydration force which is known to be an important primary barrier to fusion, or acting as a steric barrier to fusion.

In order to know how TDM alters the host cell membrane properties that tune the cellular response, Mishra et al. [53] employed membrane biophysics to assess the effects of TDM on cell membrane mechanics, lipid diffusion, and the cytoskeleton of THP-1 macrophages. Using AFM, the authors found that TDM increased cell membrane stiffness and reduced the force required for the host tethers to be extended, reducing surface tension, and bending rigidity/elastic modulus. These same authors, using two different fluorescence probes determined that TDM decreased the diffusion coefficient of lipids and reduced the recovery in ordered and disordered regions.

### Mannosylerythritol lipids

Mannosylerythritol lipids (MEL) are made of two parts, one is a hydrophilic mannopyranosyl-erythritol moiety and the other is a hydrophobic moiety containing a mixture of C6–C8 fatty acyl chains at C2' and C3' of the mannose moiety (Figure 1). In addition MEL-A possess two Ac groups each at C4' and C6' of the mannose moiety [54]. The interest in MEL-A is justified by its broad pharmaceutical applications and versatile biochemical functions [55].

The investigation of the antibacterial activity and underlying mechanism of MEL against *B. cereus* has been carried out by Shu et al. [56]. The data presented by the authors established that MEL-A induced the leakage of intracellular constituents and reduced cell viability, and using SEM and TEM showed that the treated bacterial cells exhibited apparent morphological and ultrastructural changes. All these data demonstrated that MEL-A damaged and disrupted the integrity of cell membrane. Later, the same authors showed that MEL-A was also active against *S. aureus* [57] *and L. monocytogenes* [58] through similar membrane disruption mechanisms.

The interaction between MEL-A and membranes has been also studied using simplified model membranes. In this context, Madihalli et al. [59], in their investigation of the physicochemical properties of MEL-A, used DSC measurements to study the interaction between MEL-A and DPPC, and found that in the presence of MEL-A the pretransition disappeared and the main gel to liquid crystalline phase transition widened.

Recently, Fan et al. [60] using isothermal titration calorimetry (ITC) measurements, obtained information on the binding of MEL-A to egg yolk lecithin (EYL) vesicles, and concluded that the formation of the vesicles was likely driven by the asymmetric distribution of MEL-A and phospholipids which caused the lipid curvature changes associated with the partitioning of MEL-A into the layer of PC rather than by the strong binding interaction between both molecules.

### Sophorolipids

In contrast to the above commented glycolipids biosurfactants, sophorolipids (SL) are principally produced by yeasts [61], they have remarkable physicochemical properties and biological activities and are the center of many investigations [62]. The structure of SL is formed by a sophorose moiety linked through a  $\beta$ -glycosidic bond to a long chain fatty acid (Figure 1). These biosurfactants are interesting because they can be produced in high yields and the yeast strains are nonpathogenic, which make them one of the most promising glycolipids biosurfactants.

Antibacterial activity of SL has been demonstrated against a variety of pathogenic bacteria, including *S. aureus, Lactobacillus* sp., *P. aeruginosa*, and *E. coli* [63,64]. These authors used ultrastructural observation, cell membrane permeability analysis, intracellular ATP content determination, and extracellular UV absorption detection to assess the antibacterial activity of the biosurfactant and concluded that the mechanism of antibacterial activity included the alteration of the structure of bacterial cell membrane and cell wall.

To get insight into the mechanisms underlying the SL membrane interactions, some authors have carried out experiments using biomimetic membranes and biophysical techniques. Singh et al. [65] used a single liposome assay to observe directly and quantify the kinetics of interaction of SL micelles with model membrane systems. The authors employed quantitative single particle microscopy (QSPM) and arrays of surface

tethered liposomes as model cell membranes to directly observe several successive docking events of SL assemblies on individual nanoscale liposomes. Their results revealed several repetitive docking events on individual liposomes and quantified how pH and membrane charges affected the docking of SL micelles on model membranes, providing direct evidence that acidic pH (6.5) and charges (5%), like the one found cancerous cells, is a dominant feature underlying their interaction with membranes.

Marcelino et al. [66] performed a biophysical study to reveal the molecular details of the interaction of an acidic SL with a model phospholipid membrane made of DPPC. By means of DSC it was found that SL altered the phase behavior of the phospholipid at low molar fractions, producing fluid phase immiscibility with the result of formation of biosurfactant-enriched domains within the phospholipid bilayer. FTIR spectroscopy showed that SL interacted with the phospholipid increasing ordering of the phospholipid acyl chain palisade and hydration of the lipid/water interface. SL was found to induce contents leakage in POPC unilamellar liposomes at sublytic concentrations below the CMC. The authors concluded that this SL-induced membrane permeabilization at concentrations below the onset for membrane solubilization could be the result of the formation of laterally segregated domains, which might contribute to provide a molecular basis for the reported antimicrobial actions of SL (Table 1).

# Interaction of lipopeptide biosurfactants with membranes

The number of the lipopeptide biosurfactants described in the literature is rather high [67]. For the sake of simplicity this review will focus on the four top ones, according to their physicochemical and biological properties: surfactin, lichenysin, iturin, and fengycin (Figure 3; see Table 3 for summary).

# Surfactin

The acidic lipopeptide surfactin (Figure 3) is among the most potent biosurfactants known so far. Its amphiphilic structure is formed by a seven-amino acid ring which is closed, through a lactone bonding, by a hydrocarbon tail. Surfactin is perhaps the most widely studied biosurfactant up to now. Whereas most of the studies on model phospholipid membranes have been carried out at the beginning of this century, in the last years this research has been focused mostly on biological membranes.

Surfactin is well known for its antibacterial activity which has been recently reviewed by Chen et al. [68]. This review summarizes in an illustrative figure the main extracellular and intracellular activities of the lipopeptide leading to its antimicrobial actions. In the recent years various studies have focused on the effect of surfactin on bacterial membranes. Thus, surfactin has been shown to modify bacterial target membrane composition in response to the concentration of the lipopeptide [69]. When *Bacillus subtilis* was grown in the presence of sublethal concentrations of surfactin the cells presented an adaptative response changing the phospholipid composition of the membrane. A major decrease in membrane PG was observed, whereas the proportion of PE increased up to becoming the main lipid component of the membrane. Concomitantly, the decrease in PG was compensated with a slight increase in cardiolipin, and a substantial increase in PA contents. Uttlová et al. [69] have also proposed that the increase in PA and the lower PG/PE ratio had a physiological meaning. Accordingly, the small polar headgroup of PE and PA facilitates protein insertion and, furthermore, the negative charge of phosphatidic acid can give rise to electrostatic repulsion with surfactin, preventing the interaction of the lipopeptide with the membrane. These authors considered that increasing the proportion of PA was 'the energetically cheapest way of repulsing surfactin from the membrane surface'. Interestingly, it was also found that, at difference with the phospholipid polar headgroups, the fatty acid composition was not essentially affected by surfactin.

The modifications in membrane phospholipid composition commented above were also shown to have a direct effect on surfactin-induced membrane permeabilization [69]. Thus, the membranes with similar concentrations of PG and PE, or PG, PE, and PA, were more resistant to surfactin-induced membrane permeabilization than those in which PG was the predominant phospholipid, i.e., the adaptation to growing in the presence of surfactin resulted in membranes more resistant to lysis.

Surfactin has been applied as an adjuvant for the antibiotic treatment of avian pathogenic *E. coli* with amoxicillin by Liu et al. [70]. Since amoxicillin, a  $\beta$ -lactam antibiotic, can disrupt the bacterial cell wall, the biosurfactant was used to increase the permeability of the cell membrane. Thus, these authors suggested that 'the combination of amoxicillin and surfactin could facilitate the approach of amoxicillin to cell wall', which opens a new type of applications for the biosurfactant.

The role of membrane composition on surfactin-induced membrane permeabilization has been subject of various studies in the past. Recently, the role of cardiolipin on surfactin-induced liposome contents leakage has been evaluated by Pinkas et al. [71]. One of the main conclusions of this study was that the liposome membrane composed of lipids isolated from a surfactin non-producing strain of *Bacillus subtilis* was more susceptible to surfactin-induced permeabilization. It was shown that the main difference with the surfactin-producing strain was the higher contents of cardiolipin at the expense of PE and PG, which resulted, due to electrostatic



Chemical structures of some of the cyclic lipopeptide biosurfactants cited in this review. The most frequently found R substituents are hydrogen or methyl.

repulsion, to a reduced interaction of the lipopeptide with the membrane, as hypothesized. As before [69], this study also showed that PE and PA stabilized the membrane, which was attributed to the conical shape and/or negative charge of these two phospholipids.

Surfactin has been shown to inhibit growth of *Propioni*bacterium acnes, a bacteria causing acne vulgaris [72]. It is proposed that, as in previous studies, this is a direct consequence of the interaction of the lipopeptide with the cell membrane, creating pores and leading to loss of membrane integrity. Using flow cytometry, it was shown that surfactin damaged *P. acnes* cell membrane allowing the entrance of propidium iodide into the cell. In addition, incubation in the presence of surfactin resulted in increased K<sup>+</sup> and Ca<sup>2+</sup> leakage and dissipation of membrane potential. Unilamellar liposomes with a composition resembling that of the bacterial membrane were also permeabilized by surfactin, as shown by calcein release.

The antifungal activity of surfactin, known for long time, has been recently subject of new studies. It has been reported that surfactin displays antifungal activity against *Fusarium foetens*, a fungi causing a severe disease in potatoes [73]. Among the various actions of the lipopeptide, these authors found that surfactin caused the release of nucleic acid and proteins to the extracellular fluid, indicating a damage in membrane integrity or membrane permeabilization. Furthermore, this interaction resulted in abnormal mycelial morphology, thus inhibiting mycelial growth.

In addition to its antimicrobial activity, surfactin has been reported to have strong antiviral properties. It has been reported that this antiviral actions can be due to inhibition of viral membrane fusion upon insertion of the lipopeptide into the viral envelope [74]. In this work, surfactin concentrations were low, below its hemolytic activity, and did not inhibit cell proliferation. These authors showed that surfactin, under these conditions, did not cause the disruption of the viral envelope, proposing a different mechanism from previous ones. It was shown that surfactin increased positive curvature in monolayers, due to its inverted-cone shape, proposing that it had a similar effect on the viral envelope, resulting in an impeded formation of the stalk intermediate and, thus, inhibiting viral membrane fusion [74]. A promising potential of surfactin as an antiviral agent was suggested, based on its capacity to interact with target phospholipid membranes.

The antiviral activity of surfactin has been further investigated by Yuan et al. by using synthetic analogs with a modified peptide ring but the same 16 carbon fatty acid chain [75]. The main conclusion withdrawn from the results presented in this work was that the size of the hydrophilic part of the wedge-shaped lipopeptide was related to its antiviral activity. This interesting result open the possibility of modifying surfactin structure to enhance its biological activity.

Few works have addressed, in the last years, the interaction of surfactin with mammalian cell membranes. The interaction of surfactin with the membranes of cultured cells has been recently investigated in a CHO-K1 cell line, a model mammalian cell line [76]. It was shown that, even at low concentration, surfactin altered the organization of the plasma membrane, modifying nanodomain formation, and altering membrane fluidity. The increase in fluidity is attributed to the partial removal of cholesterol from the plasma membrane by surfactin, which can also be related to alteration of lipid rafts, since cholesterol is a key component of these structures. The authors suggest, based on previous studies, that this depletion of cholesterol can affect cellular signaling pathways.

It is a well-established result that most naturally occurring surfactins display a high hemolytic activity, which was actively studied in the past. However, Fei and co-workers [77] have recently isolated a new surfactin with a 11-carbon acyl chain which displayed a low toxicity towards erythrocyte membranes. The CMC of this surfactin- $C_{11}$  was higher than the CMC of surfactin- $C_{15}$ , due to the decrease in hydrophobicity resulting in higher water solubility. It was shown that the hemolytic activity of surfactin progressively decreased with decreasing fatty acyl chains length from  $C_{16}$  to  $C_{11}$ . This is an interesting result since there is a quest for biosurfactant homologues displaying low hemolytic activity for *in vivo* applications.

As commented above, recent studies on the interaction of surfactin with phospholipid model membranes are scarce. A model system consisting of mixed monolayers of deuterated distearoylphosphatidylcholine (DSPC) and surfactin, was studied through nano-infrared microscopy and spectroscopy in combination with atomic force microscopy imaging by Kästner et al. [78]. Domain formation, of sizes below the micrometer, was shown. Within these domains, despite the bulky size of surfactin, deuterated DSPC acyl chains were shown to remain rather ordered. Using a different biophysical approach, Goussous and coworkers have studied the interaction of surfactin with a monolayer of DPPC at the air-water interface [79]. By means of non-linear sum frequency generation (SFG) vibrational spectroscopy and infrared reflection absorption spectroscopy (IRRAS) these authors proposed a mechanism explaining the temporal sequence of the events taking place upon injection of the lipopeptide in the sub-phase. Upon arrival of surfactin to the surface of the monolayer the phospholipid acyl chains tilt, due to the hydrophobic interaction with the lipopeptide. Some lipid molecules are forced to return to the water surface and, finally, there is formation of surfactin bilayers and clusters. The lipopeptide-lipopeptide interactions predominate on phospholipid-phospholipid interactions, which results in formation of pure surfactin domains and well-ordered phospholipid domains.

### Lichenysin

Lichenysins, considering their structure, belong to the surfactin family, and that is perhaps the reason why there are so few studies on this lipopeptide. However, the substitution of glutamic acid by glutamine in position 1 of the peptide ring (Figure 3) leads to distinct properties: for instance, the CMC of lichenysin is lower than that of surfactin. The mechanism of permeabilization of model and biological membranes by lichenysin has been studied in detail [80]. These authors reported that lichenysin caused rapid and extensive hemolysis of red blood cells at concentrations quite below the CMC of the lipopeptide. The faster kinetics of  $K^+$  release vs. hemoglobin release indicated and osmotic-lytic mechanism. Osmotic protection experiments indicated formation of 'pores' with a mean diameter of 34 Å. It was proposed that 'the leaky "pores" might be formed by clusters of lichenysin molecules surrounded by phospholipids, which are physicochemically feasible given its strong amphiphilic nature'. It was also shown that lichenysin permeabilized unilamellar liposomes, giving rise to release of entrapped carboxyfluorescein, at concentrations where no membrane solubilization occurred (Figure 4). Using liposomes of various compositions, it was shown that membrane lipid composition played a role in the selectivity of target membranes by lichenysin. Molecular dynamics simulations showed that lichenysin presented a rather homogeneous distribution along the z-axis but not in the xy-plane, tending to form aggregates inside the membrane, which supported the experimental leakage results.

A complementary work carried out a biophysical study on the interaction of lichenysin with various PCs of different acyl chain length [81]. The main gel to liquidcrystalline phase transition of the phospholipids was shifted to lower temperatures and broadened by incorporation of the lipopeptide, and multicomponent thermograms were observed, indicating domain formation (Figure 4). The fluid-phase immiscibility was confirmed



(a) Upon addition to POPC unilamellar vesicles (arrow) lichenysin (15 mol%) induced extensive contents leakage to the external medium (data taken from the study by Coronel et al. [80]). (b) Differential scanning calorimetry showed that, at the same concentration range (15 mol%), lichenysin gave rise to fluid-phase immiscibility, indicated by multicomponent thermograms (data taken from the study by Coronel et al. [81]).

in the corresponding phase diagrams. Lichenysin increased the interlamellar repeat distance as determined by small-angle X-ray diffraction, which was due to an increase in the thickness of the water layer between bilayers. FTIR results showed that lichenysin increased acyl chain disorder in DPPC systems, and induced dehydration of the polar region of the bilayer. This reduction in DPPC hydrogen bonding was corroborated by the results of molecular dynamics simulations. This work provided biophysical data to explain, at the molecular level, the cytotoxic, antimicrobial, and hemolytic activities of lichenysin.

Very recently it has been reported that lichenysin promoted the membrane permeabilization and the subsequent transport of nitrate and glucose in *Pseudomonas stutzeri*, which resulted in improved aerobic denitrification [82]. This effect took place both with *in situ* generated or exogenously added lipopeptide and was more effective than that of Triton X-100. Morphological features observed by SEM indicated that *P. stutzeri* maintained its shape and there were no cell deformations. This work provided evidence showing that the model membrane permeabilization commented above [80] might also take place in living microbial cells, which can have positive consequences on this microorganism.

### Iturin

Iturins are antifungal lipopeptides isolated from cultures of *Bacillus subtilis* and other closely related bacteria. Chemically they are cyclic lipopeptides composed of seven  $\alpha$ -amino acids linked to a fatty acid that differ in their amino acid composition and fatty acid chain length (Figure 3). The most important compounds of the Iturin group are iturin A-E, mycosubtilins and bacillomycins D, F and L [83]. They have a strong antifungal activity against fungi and bacteria pathogenic to humans and plants.

Four variants of iturin A and a new lipopeptide were isolated and identified from *Bacillus subtilis* N-2 [84]. Morphological changes of *Pseudomonas putida* were observed after lipopeptide treatment. Bacterial cells showed edges, rough surface, and folds. In addition, the growth of *P. putida* was significantly inhibited in the presence of these lipopeptides by disrupting cell membrane permeability.

Bacillomycin D is an antifungal agent of the iturin group (Figure 3). In this work it was isolated from *Bacillus velezensis HN-2* to study its mechanism of action [85]. *Colletotrichum gloeosporioides* (Penz.) treated with bacillomycin D displayed marked morphological changes: distortion and depression of the outer coat and inner membrane of the *C. gloeosporioides* (Penz.). Scanning and transmission electron microscopy observations showed different cell damages in cells treated with bacillomycin D, e.g., disruption of the cell wall and cell membrane and breakdown of inner organelles including nuclear lysis. In this work [85] the effect of bacillomycin D as antifungal activity is discussed based on its amphiphilic structure, which differs from other fungicides, first causing cell membrane perforation and then cytosol leakage.

*Bacillus amyloliquefaciens* NCPSJ7 showed potential fungicidal activities for the effective control of fungal infection by *Fusarium oxysporum f.* sp. *niveum* [86]. Different genes closely related to lipopeptide synthesis were detected in *Bacillus amyloliquefaciens* NCPSJ7 [87],

corresponding to iturin, surfactin, fengvcin, and bacillomycin. C14-iturin A was identified and isolated from Bacillus amyloliquefaciens NCPSJ7. In the presence of C14iturin A the mycelial structure was affected as seen by electron microscopy techniques, as well as concomitant modifications inside the cells, such as swollen mitochondria, enriched glycogen, and increased vacuoles. These results lead to an increase of membrane permeability and fungal growth inhibition. It is known that ergosterol is an essential component of fungal cell membranes that determines membrane properties such as fluidity, permeability and membrane bound enzymes and transporters [88,89]. In the previously mentioned work [87], the ergosterol content increased in the mycelium treated with C14-iturin A, which could be explained by a positive regulation of ergosterol synthesis as a fungal response to the stress of increased membrane permeability by the presence of C14-iturin A.

In response to the coronavirus pandemic, Shekunov et al. [90] have sought alternative strategies for antiviral therapy. These authors have studied the effects of cyclic lipopeptides on liposome fusion induced by calcium, by PEG-8000, and by a SARS-CoV-2 fusion peptide fragment. The cyclic lipopeptides used were anidulafungin, caspofungin, aculeacin A, iturin A, fengycin, surfactin, mycosubtilin, polymyxin B, daptomycin, syringostatin A, and syringotoxin B. Three different lipid mixtures of lipids were used to make the liposomes: DOPC/DOPG/ CHOL, DOPC/CHOL and POPC/SM/CHOL. The liposome fusion was monitored by a calcein leakage fluorescence assay. They concluded that the ability of lipopeptides to inhibit membrane fusion depended on their membrane composition (DOPC/DOPG/CHOL vs DOPC/CHOL) not on the type of fusion trigger (CaCl<sub>2</sub> vs PEG-8000). Membrane vesicles composed of POPC/ SM/cholesterol were used to mimic SARS-CoV-2 fusion with membranes mediated by mediated by the fragment 816-827 of the SARS-CoV-2 fusion peptide. Antiviral activity of lipopeptides against SARS-CoV-2 was also studied. Only aculeacin A, anidulafungin, iturin A, and mycosubtilin A showed both antifusogenic and antiviral properties. They demonstrated that the inhibitory effect of the cyclic lipopeptides under study on membrane fusion can be related to the disordering of membrane lipids, the induction of positive curvature stress and enhancing raft formation. They finally concluded that aculeacin A, anidulafugin, iturin A, and mycosubtilin act as potent membrane fusion inhibitors combating SARS-CoV-2, and hence they could be used to prevent and fight against SARS-CoV-2 infection.

### Fengycin

Fengycin is a cyclic lipopeptide produced by *Bacillus subtilis*, which is commonly found in soil and aquatic environments (Figure 3). Lipopeptides are widely used as biosurfactants in the food, pharmaceutical, chemical, cosmetic, and even oil, and gas industries. Fengycin, like

other lipopeptides produced by *B. subtilis*, is a biosurfactant that performs the same function as a synthetic surfactant. However, it is biodegradable and less toxic, so it can be used as a sustainable substitute for synthetic surfactants.

It has been reported [91] that *Bacillus subtilis* strain EA-CB0015 produces the cyclic polypeptides iturin A, fengycin C and surfactin, and inhibits Mycosphaerella finensis growth, a fungus that causes black Sigatoka disease in banana plants. Among the above lipopeptides only iturin A and fengycin C inhibit mycelial growth and ascospore germination. The interaction of fengycin C with DPPC model membranes was carried out to explain its antifungal activity through membrane permeabilization [91]. DSC was used to study the thermotropic phase transitions of the lipid membrane. The presence of fengycin C in a DPPC membrane abolished the transition from the gel lamellar phase  $(L_{\beta'})$  to the rippled gel phase  $(P_{\beta'})$ . It also broadened the transition from the gel phase  $(P_{\beta'})$  to the liquidcrystalline phase  $(L_{\alpha})$ . This result indicated loss of cooperativity due to disruption of lipid packing by intercalation of the acyl chain of the lipopeptide among the DPPC molecules [92]. In addition, two more endothermic components appeared because of the lateral phase segregation of a DPPC/fengycin complex which could facilitate membrane permeabilization. Studies of fluorescent polarization of diphenylhexatriene (DPH) and FTIR spectroscopy in the carbonyl absorption region indicated that fengycin was located close to the polar headgroups of DPPC since it did not affect the fluorescent of DPH (a probe of core of the lipid bilayer) and it dehydrated the carbonyl groups of DPPC. All these results help to explain the antifungal effect of fengycin due to its membrane-disrupting action that can trigger membrane permeabilization and, ultimately, cell lysis [91].

It was established that fungal membranes with low levels of ergosterol were more sensitive to antifungal lipopeptides and specifically to fengycin. In order to evaluate the effect of the presence of fengycin in membranes containing ergosterol, Mantil et al. [93] used model membranes of DOPC/DPPC (3:1) containing up to 12 mol% ergosterol. Different concentrations of fengycin were added to these membranes to obtain images by total internal reflection fluorescence microscopy (TIRFM). This technique allows visualizing and differentiating between ordered  $(L_{\beta}/L_0)$  and disordered  $(L_{\alpha}/L_{d})$  domains in a lipid membrane. In general, the presence of fengycin caused a decrease in the number of L<sub>0</sub> domains, but this effect was attenuated with increasing ergosterol content in the membrane. An increased ergosterol content would decrease the activity of fengycin. Therefore, ergosterol would act buffering the insertion of fengycin into the membrane, retaining ordered packing [93].

A remarkable result was obtained by Zakharova et al. [94] using planar lipid bilayers to study the effect of fengycin in one side of a membrane. The planar lipid membranes were composed of POPC/POPE/POPG/ergosterol to mimic composition of target fungal cell membranes. They found that the addition of fengycin leads to the formation of single-ion channels presenting weak cation selectivity. The formation of pores was accompanied by oligomerization of fengycin molecules, and they concluded that at least a dimer is needed to form a single channel. Two different models are being used to explain the activity of the lipopeptide on a membrane. One when the lipopeptide acts as a detergent, solubilizing the membrane, and another when the lipopeptide is introduced into the lipid membrane, forming pores, altering the permeability of the membrane. The results of Zakharova et al. [94] clearly support the antifungal activity of fengycin through membrane permeabilization caused by ionic pore formation.

The interaction of fengycin with complex membrane models was addressed by Mantil et al. [95]. For this purpose, Langmuir lipid monolayers were prepared from lipid extracts from fungal (Alternaria solani and Fusarium sambucinum), and from oomvcetal (Pythium sulcatum) molds to study the incorporation of fengycin to these membranes. Determination of critical pressures of insertion of fengycin as a measure of the affinity of fengycin to these membranes revealed that it was negatively correlated with ergosterol content in the membranes of the three molds. This result was interpreted as that the presence of ergosterol in the membrane decreases the affinity for fengycin. From these lipid extracts, liposomes were prepared to study fengycin-induced leakage, as a measurement of the release of the fluorescent probe calcein. Calcein leakage induced by fengycin was negatively correlated with ergosterol content, i.e., membranes with higher ergosterol content were more resistant to leakage caused by fengycin. Other interesting and relevant correlations with the lipid composition of the membrane were drawn out from this work (Table 2).

Similar results were obtained by using TIRFM to evaluate changes in domain distribution by the addition of fengycin to supported lipid bilayers made from the same lipid extracts [96]. The presence of ergosterol in the membranes promotes lipid phase ordering, buffering the effects of fengycin. The same conclusion was obtained in DOPC/DPPC/ergosterol membranes (see above) [93].

The antibacterial activity of cyclic lipopeptides against various bacterial pathogens had been attributed to iturins and surfactins, but not much information was available on the bactericidal properties of fengycin [97]. Recently, Medeot et al. [98] isolated fengycin from *Bacillus amyloliquefaciens* as the lipopeptide with the most antifungal and antibacterial activity of those produced

#### Table 2

Correlation of some lipid parameters with the critical pressures of insertion (CPI) and fengycin-induced leakage. Sign of the correlation coefficient (r) is shown only when  $r \ge |0.76|$ . Data obtained from the study by Mantil et al. [95].

Lipid	CPI	Leakage
Ergosterol Phosphatidylcholine Phosphatidylethanolamine Phosphatidylglycerol Average degree of unsaturation Lipids acyl chain length	Negative Negative Positive – Negative Positive	Negative Negative – Positive –

by this bacterium. It was observed that fengycin had antibacterial activity against the pathogens *Xanthomonas axonopodis* and *P. aeruginosa*. From atomic force microscopy images, changes on bacterial cell surfaces were observed, from clear alterations in bacterial cell morphology at low fengycin concentration to total cell damage at higher fengycin concentrations. Besides, measurements of K<sup>+</sup> fluxes, determined by single channel flame photometry, showed that the permeability to K<sup>+</sup> ions was lost in the presence of fengycin. Furthermore, the toxicity of fengycins on mammal cells was evaluated on lung fibroblasts. The viability of these cells was not altered by the fengycin treatment [98]. The importance of this work is that it presents a new antibiotic agent that could be used in clinical practice.

RL and fengycins are well-known biosurfactants with antifungal activities against phytopathogenic fungi by direct interaction with the fungal lipid membranes. Botcazon et al. [24] addressed the differences in both molecules with respect to their antifungal action. RL promote asperities emergence and hyphal fusions whereas fengycins promote vacuole fusions and autophagy. The protective effect of ergosterol on the fungal membrane against the addition of fengycin has been addressed above [93–96]. The effect of the presence of ergosterol in the fungal membrane has opposite behavior against the addition of RL and fengycins. Thus, ergosterol increases RL effects, whereas ergosterol buffers fengycins effects [24].

It was previously suggested that the formation of fengycin aggregates in the lipid membrane surface could play a key role in cell disruption. Molecular dynamics simulations were used to study properties such as specificity, selectivity, and structure of these fengycin oligomers [99]. The effect of fengycin on bacteria and fungi was addressed. Fungal membranes were simulated by constructing POPC lipid membranes, while bacterial membranes were simulated using POPE/POPG (2:1) mixtures, both in the presence of fengycin. The presence of fengycin caused a decrease in the order parameters of the lipid acyl chains, indicating an increase in membrane disorder in both membranes. The radial lateral distribution function between fengycin peptide ring and the lipid head group showed that fengycin had a preferential interaction with POPE but not with POPG or POPC. This result was explained by glutamates preferential packing with the POPE amines by electrostatic interactions. However, the lateral radial distribution function between the fengycins clearly showed that fengycin aggregation was observed on both membranes, but the largest aggregates were only stable on the POPC membranes (representative of fungal membranes). They concluded that the formation of fengycin oligomers could explain the selectivity of fengycin towards fungi compared to bacteria [99].

Cholesterol is present in mammalian cells but not in fungal cells. It was shown that cholesterol reduced

Table 3

Lipopeptide biosurfactants	System	Effects	Reference
Surfactin			
	Mixed phospholipids	Membranes with similar concentrations of PG and PE, or PG, PE, and PA, were more resistant to surfactin-induced membrane permeabilization than those in which PG was the predominant phospholipid.	[69]
	Cardiolipin, PE, PG, PA	Liposome membrane composed of lipids isolated from a surfactin non-producing strain of <i>B. subtilis</i> was more susceptible to surfactin-induced permeabilization.	[71]
	Mixed phospholipids	Unilamellar liposomes with a composition resembling that of the bacterial membrane of <i>P. acnes</i> were permeabilized by surfactin.	[72]
	Deuterated DSPC	Domain formation, of sizes below the micrometer, was shown. DSPC acyl chains remained ordered.	[78]
	DPPC monolayers	Lipopeptide-lipopeptide interactions predominate on phospholipid-phospholipid interactions, which results in formation of pure surfactin domains and well-ordered phospholipid domains.	[79]
Lichenysin			
	POPC, POPE, POPG	Permeabilization of unilamellar liposomes, giving rise to release of entrapped carboxyfluorescein, at concentrations where no membrane solubilization occurred.	[80]
	PCs of various acyl chain lengths	The main gel to liquid-crystalline phase transition of the phospholipids was shifted to lower temperatures and broadened by incorporation of the lipopeptide.	[81]
Iturin		Manoomponom normogramo maioacoa domain termation.	
	DOPC, POPC, DOPC, DOPG, SM, cholesterol	The ability of the lipopeptide to inhibit membrane fusion depended on their membrane composition not on the type of fusion trigger (CaCl <sub>2</sub> or PEG-8000).	[90]
Fengycin	DPPC	Disruption of lipid packing by intercalation of the acyl chain of the lipopeptide among the DPPC molecules.	[91]
	DOPC, DPPC, ergosterol	Fengycin caused a decrease in the number of $L_0$ domains. Errosterol in the membrane attenuated this effect.	[93]
	POPC, POPE, POPG, ergosterol	Addition of fengycin led to formation of single ion channels presenting weak cation selectivity in planer lipid bilayers	[94]
	Lipid extracts from fungal molds	The presence of ergosterol in the membrane decreased the affinity for fengycin. Membranes with higher ergosterol content were more resistant to leakage caused by fengycin.	[95]
	POPC, POPE, POPG	The presence of fengycin caused a decrease in the order parameters of the lipid acyl chains, indicating an increase in membrane disorder in both membranes. Formation of fengycin oligomers could explain the selectivity of fengycin towards fungi compared to bacteria.	[99]
	POPC/cholesterol MD simulations	Subtle alteration in the aggregation of fengycin was observed in the presence of cholesterol. In the absence of cholesterol, a greater diversity was observed both in the number of fengycin–fengycin contacts and in the number of different aggregates.	[100]

membrane leakage induced by the addition of fengycin. Therefore, molecular dynamics simulations was performed to show the effect of the presence of cholesterol in the membrane on the formation of fengvcin oligomers [100]. The simulated system was a mixture of POPC/cholesterol (4:1) in the presence and in the absence of fengycin. Only a subtle alteration in the aggregation of fengycin was observed in the presence of cholesterol; however, fengycin was more likely to form a single large aggregate. On the contrary, in the absence of cholesterol, a greater diversity was observed both in the number of fengycin-fengycin contacts and in the number of different aggregates. In addition, the presence of cholesterol limited membrane disorder and reduced the ability of the aggregates to fold the membrane. All these results suggest that the presence of cholesterol reduces the ability of fengycin to perturb the membrane [100]. Thus, the presence of cholesterol in mammalian cells would protect the cell membrane against the incorporation of fengycin in the membrane (Table 3).

# Summarizing remarks

Biosurfactants have diverse potential biological functions which, given their amphiphilic properties, are determined by their ability to interact with biological membranes. The study of the interaction between biosurfactants and a variety of biological membranes of different origin, including bacterial, fungi, viral, plant and animal ones, has revealed that biosurfactants are able to disrupt and permeabilize the membrane leading to a decrease of cell viability. Many scientific studies have been devoted to investigating the interaction between biosurfactants and model membrane systems to understand the molecular mechanisms underlying the effect of biosurfactants on the different types of biological membranes. Several experimental techniques including calorimetry, infrared and fluorescence spectroscopy, nuclear magnetic resonance, X-ray diffraction or microscopy, together with simulation by MD have been used to get insight into the molecular basis of the effects of biosurfactants on the lipidic component of membranes. Information on fluidity, order, dynamics, thickness, hydration, thermotropic transitions of the bilayer, and location of the biosurfactants in the membrane have been obtained from the study of model systems with a variety of different composition and complexity. These investigations are contributing to figure out the molecular basis of the biosurfactant membrane interaction, and to obtain a more comprehensive view of the effects of these compounds on biological membranes that explain their biological activities. In this review, we have analyzed and discussed to some extent the most recent investigations and perspectives with the aim to update the present understanding and advancements on the interaction between biosurfactants and model and biological membranes.

# **Declaration of competing interest**

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

# Data availability

No data was used for the research described in the article.

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