

From sustainable fermentation to smart nanocarriers: Sophorolipid-phospholipid hybrid vesicles with antibacterial and antiproliferative activity

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ABSTRACT

Sophorolipids (SLs) are microbial glycolipids with amphiphilic properties that have attracted attention as sustainable bioactive molecules with potential applications in antimicrobial and anticancer therapies. In this study, we evaluated how the degree of purification (crude, partially purified and purified) and the structural type of SLs (diacetylated lactonic) influence their physicochemical characteristics and biological activities, and we further investigated their performance when formulated into hybrid phospholipid-based vesicles. Specifically, the enrichment in lactonic C18:1 congeners (>90% in the purified SLs) through consecutive purifications steps led to lower critical micelle concentration (CMC) values and enhanced the stability of the resulting formulations in terms of Z-average and polydispersity index (PDI). Purified SLs showed strong antimicrobial activity against Gram-positive bacteria with minimum inhibitory concentrations (MIC) comparable to gentamicin and lower activity against Gram-negative and *Candida* spp. In addition, purified SLs demonstrated marked antiproliferative effects on two selected breast cancer cell lines (SK-BR-3 and DA-MB-231), with IC₅₀ values in agreement with those reported in the literature. The formation of hybrid vesicles composed of purified SLs and dimyristoylphosphatidylcholine (DMPC) retained the antimicrobial and cytotoxic activity with the respect to free SLs, supporting the potential application of these delivery systems. Overall, the results highlighted the critical role of purity and structural type in determining the functional properties of SLs together with the exploitation of vesicular formulations in enhancing their applicability. These findings provide a foundation for the rational development of SLs-based therapeutics and encourage further *in vivo* evaluation of their efficacy and safety.

1. Introduction

Biosurfactants are surface-active natural compounds mainly produced by microorganisms (bacteria, fungi and yeast) as secondary metabolites. They can be constituents of the microbial cell surface or being excreted in the environment to exert a selective function in terms of microorganism survival, since they are involved in several mechanisms such as nutrient transport, cell–host interactions, quorum signalling and competitive killing actions [1,2].

Recently, biosurfactants have emerged as a novel class of natural surfactants having relevant advantages over most of the synthetic ones. In addition to their production from renewable resources, these advantages include favourable cyto- and ecotoxicological profiles, due to the reduced toxic effect on biological membranes, as well as the high rate of environmental biodegradability [3].

For these reasons, several types of biosurfactants have been investigated in a wide range of technological applications in the pharmaceutical, cosmetics, food and agriculture fields, thanks to their surface

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active/emulsifying ability as well as their intrinsic functional properties (e.g. antimicrobial activity) [4].

Among them, sophorolipids (SLs) are one of the main classes of microbial glycolipid biosurfactants, which are mainly produced by the yeast species belonging to *Starmerella* and *Candida* genera. SLs can be produced by either submerged (SmF) or solid-state (SSF) fermentation techniques. Production through SmF has been more extensively studied and developed to a commercial scale [5], typically employing first-generation substrates such as vegetable oils and glucose, and more recently, various waste hydrolysates [6]. In contrast, SSF remains at a lower technological readiness level and is generally focused on the bioconversion of solid residues or by-products, such as oil cakes from the food industry [7]. The interest in SLs relies on their potential scalability to industrial production since they can be obtained with high yields (about 300 g L⁻¹) via a fermentation process. Chemically, they are composed of a disaccharide sophorose unit linked to a hydroxylated fatty acid (mainly 18:1 in acid or lactonic form) via a β -1,2-glycosidic bond. Generally, the lactonic forms provide compounds with a greater ability to decrease surface tension and better antimicrobial activity than the corresponding acid form, despite having a lower solubility in water [8,9].

In addition to the surface and emulsifying properties [10], SLs are also attractive thanks to their intrinsic antibacterial, antiviral [11] and anticancer [12] activities, which can be exploitable for their applications in the pharmaceuticals, cosmetic and food fields [8,13]. At this extent, the design of novel formulations for the delivery of SLs is desirable to enlarge their use. Both native and chemically modified SLs have been employed in the formulations of emulsions [14,15], microemulsions [16,17], nanoemulsions [18,19], films [20,21] and used as coating material for implants [22] with antimicrobial properties. The parental administration of SLs as additives or bioactive ingredients can benefit from the development of novel nanosystems [23,24]. SLs have been already employed as capping and surface-functionalizing agents for the synthesis of several metallic nanoparticles in different studies. However, limited literature is available regarding their incorporation into hybrid nanosystems formed by polymers or lipids. These hybrid nanosystems could act as effective carriers for SLs by enhancing their solubility, stability and biological activities after a parenteral administration. Due to the lipid nature, it is interesting to investigate the feasibility of preparing hybrid systems composed of SLs and phospholipids. In a study, Marcelino et al. investigates the interaction of an acidic SL biosurfactant with 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) model membranes [25]. In another study, piperidine-loaded liposomes were functionalised with SLs to improve their stability and dispersibility, demonstrating also a sustained release of the drug [26]. Apart from these studies, no other hybrid systems formed by the incorporation of SLs into phospholipid-based vesicles are reported in the literature and no one focused on the effect of the purity grade of SLs and on the biological activity of the prepared nanosystems. Therefore, the aim of the present study is to investigate the feasibility of preparing hybrid nanosystems formed by SLs, obtained by a solid-state fermentation process from *Starmerella bombicola* at different purity grade, and phospholipids using 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) as a model. Moreover, the antimicrobial activity toward Gram-positive, Gram-negative bacteria, fungi and the antiproliferative effect on human breast cancer cells of the prepared hybrid nanosystems was tested to assess the effectiveness of the formulations and provide smart nanoplateforms for the targeted delivery.

2. Materials and methods

2.1. Materials

All solvents (n-hexane, ethyl acetate, chloroform and methanol) used for the extraction, purification and characterization were obtained from

Merck Life Science S.L.U. (Madrid, Spain) and were analytical grade. Dimyristoylphosphatidylcholine (DMPC) was purchased from Avanti Polar lipid, inc. (Alabaster, AL, USA). Gentamicin and amphotericin B were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mueller Hinton Broth Oxoid™ (MHB) and Sabouraud Dextrose Broth Oxoid™ (SAB) were purchased from Thermo Scientific (Milan, Italy). Dulbecco's Modified Essential Medium (DMEM), fetal bovine serum (FBS), and 1% penicillin-streptomycin were purchased from Gibco, Life Technologies (Carlsbad, CA, USA).

2.2. Production and extraction of crude sophorolipids

SLs were produced by solid-state fermentation (SSF) as previously described by Rodríguez et al. [27]. Briefly, a mixture composed of wheat straw, residual sunflower oil cake, and sugar beet molasses was sterilized and inoculated with *Starmerella bombicola* ATCC 22214, then fermented in a 22-L bioreactor for 6 days. Crude SL was obtained by solid-liquid extraction as described by Oiza et al. [28]. The dried fermented solid material was extracted twice with ethyl acetate (1:10 v/w ratio, 1h, 200 rpm, room temperature). Crude SL was obtained by vacuum evaporation at 40 °C of mixed extracts after n-hexane washing. Crude SL extract was stored at -20 °C.

2.3. SL purification and characterization

Both partially purified SL and purified lactonic SL were obtained through adsorption chromatography using a 60 cm × 6 cm column packed with 60 Å, 230-400 mesh silica. Mobile phases were methanol and chloroform.

A two-step purification was performed using 1L of 10% v/v methanol in chloroform and a second phase of 20% v/v methanol in chloroform. Fractions were collected and characterized through HPLC-MS. All fractions containing no SL were discarded and those with SL and low free fatty acids content were combined to obtain partially purified SL.

Multi-step purification was performed starting at 5% v/v methanol in chloroform and increasing methanol percentage in 5% increments up to a maximum of 20% v/v. 500 mL of each phase was used per step. All fractions were characterized through HPLC-MS and those containing only C18 lactonic diacetylated SL were combined as purified SL.

Characterization of crude, partially purified and purified SL was performed by HPLC-MS and HPLC-UV as described by Ingham et al. [29] and adapted by Oiza et al. [28]. Briefly, a Phenomenex nucleopore C18 column (3 µm 120 Å 100 × 4.6 mm) was used. 0.01% v/v formic acid was added to both mobile phases, water, and acetonitrile. A slow ramp of 45 min from 30 to 90% acetonitrile was applied. For mass analysis (MS), a Q-TOF (timsTOF Pro 2, Bruker, USA) was employed using the same separation method and run on negative mode with a scanning range of 50-1000 m/z. SLs structures were inferred from the HPLC-MS masses following the reported literature [30].

2.4. Surface tension measurements on SL mixtures

Surface tension of different concentrations of SL mixtures in ultrapure water was measured at 25 °C using force tensiometer "DCA-100 (First Ten Angstroms)" according to the "Du Noüy ring" method. Three independent measurements were carried out for each concentration of SL mixtures. The critical micelle concentration (CMC) and surface tension at CMC (γ CMC) values were calculated using the segmental linear regression approach (GraphPad Prism 6 software).

2.5. Fluorescence measurement on SL mixtures

Fluorescence analyses were performed using a FluoroMax Spectrofluorometer (Horiba Scientific). Pyrene was dissolved in methanol at the concentration of 2 µM and 3 µL were added to the aqueous surfactant solutions at different concentration. The excitation wavelength for

pyrene was 334 nm. Spectra were recorded at room temperature in the range between 200 and 700 nm with a 2.5 nm excitation slit and a 2.5 nm emission slit. The ratio of peak intensity I ($\lambda = 372$ nm) to peak intensity III ($\lambda = 384$ nm) of the emission spectrum of pyrene was plotted against surfactant concentrations. CMC values were calculated from the centre of the sigmoid by fitting the experimental data using the Boltzmann nonlinear regression according to the following equation:

$$Y = \frac{\text{bottom} + (\text{top} - \text{bottom})}{1 + 10^{[(\text{LogCMC} - x) / \text{hill slope}]}}$$

where top and bottom are the plateaux of the curve in the unit of Y-axis and hill slope is the steepness of the curve. Three independent measurements were carried out for each concentration of SL mixtures.

2.6. Hybrid phospholipid-SLs vesicles

Vesicles were prepared using the thin-layer hydration method. Briefly, the required amounts of DMPC as phospholipids and SLs were dissolved in chloroform, then dried under vacuum and rehydrated with ultrapure water at 55 °C. Then, the liposomal dispersions were passed through a 0.2 μm membrane filter ten times using a mini-extruder (Avanti polar Lipids) and stored at 4 °C. The final concentration of DMPC in all liposomal dispersions was 5 mg/mL. SLs (crude, partially purified and purified) were added in the formulation at the ratio of 1:0.2, 1:0.6 and 1:1 with respect to the weight of DMPC.

2.7. Dynamic light scattering

DLS analyses were performed using a Malvern Zetasizer nano (Malvern instrument Worcestershire, Malvern, UK), with a detection angle of 173°. The hydrodynamic diameter of vesicles (Z-average) and the width of the particle size distribution (polydispersity index, PDI) were measured at 25 °C after an equilibration time of 180 s. Samples were analysed after their preparation and at different timepoints (until 63 days) to assess the physical stability. Analyses were conducted in triplicate.

2.8. Cryo-TEM

Vitrified specimens were prepared by placing 4 μL of a sample on a Lacey Carbon TEM grid (copper, 400 mesh). Each sample was blotted to a thin film and immediately plunged into liquid ethane in the Leica EM GP cryo-workstation. The grids were viewed on a JEOL 2011 transmission electron microscope operating at an accelerating voltage of 200 kV. The microscope was equipped with a 626 Gatan cryoholder, and the samples were maintained at -182 °C during imaging. Electron micrographs were recorded with the Digital Micrograph software package (Gatan) under low electron dose conditions, to minimize electron beam radiation. Images were recorded on a Gatan RIO16 CMOS camera. The images were imported in ImageJ, where they were calibrated and analysed in terms of Feret diameter.

2.9. High-resolution ultrasound spectroscopy (HR-US)

Ultrasonic analyses were carried out using an HR-US 102 high-resolution spectrometer (Ultrasonic Scientific, Dublin, Ireland) as reported in Ref. [31]. The differential relative ultrasound speed was measured over temperature in the range of 5–50 °C and at the rate of 1 °C/min. The transition temperatures were determined from the minimum of the first-derivate of sound speed signal [32]. All measurements were performed in triplicates.

2.10. Antimicrobial studies

Following the recommendations of the Clinical and Laboratory

Standard Institute (CLSI) ([33]), the broth microdilution method was applied to determine the *in vitro* minimum inhibitory concentrations (MICs, $\mu\text{g/mL}$) and the minimum bactericidal concentrations (MBCs, $\mu\text{g/mL}$). SLs under investigation was initially prepared at the highest concentration to formulate stock solutions. These stock solutions were subsequently diluted at a ratio of 1:10 using Cation-Adjusted Mueller Hinton Broth. This procedure resulted in a gradient of concentrations ranging from 2048 $\mu\text{g/mL}$ to 2 $\mu\text{g/mL}$ within the wells, achieved by performing two-fold serial dilutions in the designated testing medium. The stock solutions were prepared using pure DMSO as diluent. The following bacterial and fungal strains, that available as freeze-dried discs, from the American Type Culture Collection (ATCC, Rockville, MD, USA) were screened: *Bacillus cereus* (ATCC 10876), *Bacillus subtilis* (ATCC 6633), *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 6538p, ATCC 25923, ATCC 29213, ATCC 43300), *Acinetobacter baumannii* (ATCC 19606), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 27853), *Candida albicans* (ATCC 10231, ATCC 90028), *Candida krusei* (ATCC 6258), *Candida glabrata* (ATCC 15126); and some strains derived from clinical isolation; *Enterococcus faecalis* BS, *Staphylococcus aureus* BS, *Escherichia coli* BS (ESBL), *Klebsiella pneumoniae* BS. The clinical isolate was collected from patients at the Department of Biomedical Science and Human Oncology intensive care unit, University of Bari, Italy. The isolation and identification procedures were conducted in the Hygiene Section of the Department, using conventional physiological and morphological methods (API systems). To preserve the cell integrity, all bacterial strains were stored in cryovials containing suitable culture medium at -80 °C. Pre-cultures for each bacterial strain were prepared using Mueller Hinton Broth (MHB) and incubated for 3 to 5 h at 37 °C. For fungal strains, Sabouraud Dextrose Broth (SAB) was used as growth medium, and cultures were incubated for 24–48 h at 30 °C to allow adequate yeast proliferation. Following the CLSI M7-A9 guidelines, the turbidity of the bacterial cell suspension was adjusted to match the 0.5 McFarland Standard using a spectrophotometer (OD625 nm readings between 0.08 and 0.10). This standardized suspension was then diluted further (1:100) using MHB to achieve a concentration of $1\text{--}2 \times 10^6$ CFU/mL. In the case of yeasts, the inoculum density was standardized by adjusting the cell suspension to a final concentration of approximately $1\text{--}5 \times 10^3$ CFU/mL, as recommended by the CLSI M27-A3 protocol. Aliquots of 200 μL from the final inoculum were introduced into each well. Control wells containing only the inoculated broth were also prepared to monitor bacterial growth. The plates were incubated at 37 °C for 24 h, with the minimum inhibitory concentration (MIC) values determined as the lowest concentration of the compounds at which no visible bacterial growth was observed. Each experiment was performed three times in duplicate. Gentamicin and amphotericin B were used as the standard reference drugs for bacteria and fungi, respectively. Moreover, the antibacterial efficacy of DMSO alone (which served as a solvent for the studied compounds) was assessed; hence, solvent volumes below 5% concentration were utilized throughout the experiments. The liposomal preparations were tested for antimicrobial activity using the same broth microdilution method described above. Briefly, the liposomal formulations containing SLs were diluted in sterile MHB (for bacteria) or SAB (for fungi) to obtain two-fold serial dilutions, maintaining the same concentration range (2048–2 $\mu\text{g/mL}$). The inocula were prepared and standardized as described, and 200 μL of the bacterial or fungal suspension were added to each well containing the vesicles dilutions. Control wells with empty vesicles (without SLs) were also included to rule out any intrinsic antimicrobial effect of the vesicular system. For minimum bactericidal concentration (MBC) evaluation, 10 μL from clear wells were plated onto Mueller–Hinton agar and incubated for an additional 24 h. MBC was defined as the lowest concentration causing $\geq 99.9\%$ reduction in viable cells compared to the initial inoculum. All assays were conducted in duplicate and repeated independently three times to ensure reproducibility.

2.11. Antiproliferative assay

Human MDA-MB-231 (ER-/PR-/HER2-) and SK-BR-3 (ER-/PR-/HER2+) cells were obtained from American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's Modified Essential Medium (DMEM, Gibco, Life Technologies), supplemented with 10 % fetal bovine serum (FBS, Gibco, Life Technologies) and 1% penicillin–streptomycin (Gibco, Life Technologies). Cells were cultured at 37 °C under humidified atmosphere with 5 % CO₂. Cell viability was evaluated by seeding 7.000 cells/well (for MDA-MB-231 cell line) or 1×10^4 cells/well (for SK-BR-3) in 96-well plates in complete medium. The day after, cells were treated with crude, partially purified, and purified SLs or the liposomal formulation (1–200 µg/mL, in DMEM plus 10% FBS) for 24 h. Untreated cells served as a control, cells treated with the same volume of the highest concentration served as a vehicle; cell-free wells as blanks. Cell viability was determined using a MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide, Sigma Aldrich, St. Louis, MO] assay, as described by Wang et al. [34]. Each condition was tested with 8 replicates. Data were analysed using GraphPad Prism 6 software. Half-maximal inhibitory concentration (IC₅₀) values were determined by fitting through nonlinear regression the concentration–effect data by the following model:

$$Y = \frac{100}{(1 + 10)^{((\text{Log}(IC_{50} - X))^{\text{hill slope}})}}$$

where Y is the cell viability (%), X is the concentration, and hill slope is a parameter related to the curve steepness.

2.12. Statistical analysis

Surface tension, CMC, and γ CMC from tensiometric analysis, CMC from fluorescence, Z-average and PDI from DLS analysis, sol-gel transition temperatures from HR-US spectroscopy were reported as mean $\pm$ standard deviation. Volume-based Feret diameter distributions were built up and the median and interquartile range (IQR) were calculated using Minitab (v.18.1, Minitab Inc., State College, PA, USA). IC₅₀ values were estimated by fitting all individual MTT assay experimental data using nonlinear regression. The 95% confidence intervals were calculated using the standard method for parameter estimation

with nonlinear regression [35].

3. Results

3.1. Production and purification of SLs mixtures

SLs production through SSF using residual sunflower oil cake, sugar beet molasses, and *Starmerella Bombicola*, exhibited the expected trends in reference to biological activity (measured as oxygen uptake rates, OUR), temperature, and yield [27]. OUR reached peak values between 3.6 and 4 mg O₂/g dry matter, and a crude SL extract yield of 0.165 g/g DM was obtained after 6 days.

The crude SLs extract was analysed by HPLC–MS following hexane washing. Both acidic and lactonic forms of SLs were detected. As expected for wild-type *S. bombicola*, the main congener was the lactonic diacetylated C18:1 SL, which eluted at 25 min (Fig. 1). Quantification by HPLC–UV indicated that this congener represented approximately 30% of the total SLs mixture, with the remaining constituents being a combination of other SLs congeners and residual fatty acids from the fermentation.

A two-step purification procedure yielded six 200-mL fractions. Fraction 4 displayed a dense, honey-like consistency and HPLC–MS analysis confirmed the presence of both acidic and lactonic SL. Notably, the UV signal corresponding to the main lactonic diacetylated C18:1 congener increased to ~60% of total SLs, accompanied by a reduction in residual fatty acid content. This fraction was designated as the partially purified SL sample.

A subsequent multi-step purification procedure produced fourteen 100-mL fractions. Fractions 5–9 contained a whitish solid, which HPLC–MS identified as isolated lactonic SLs. These fractions were, then, pooled to obtain the purified lactonic SL sample. Four main chromatographic peaks were observed (Fig. 1), all corresponding to lactonic diacetylated SL congeners differing only in the fatty acid tail saturation: C18:2, C18:1 (two isomers), and C18:0. These samples contained >99% lactonic SLs, with the C18:1 diacetylated lactonic congener being the predominant form and no detectable residual fatty acids.

In summary, three SLs samples were prepared for use in subsequent experiments: i) crude SL, containing SLs and residual fermentation-derived fatty acids without any purification process; ii) partially

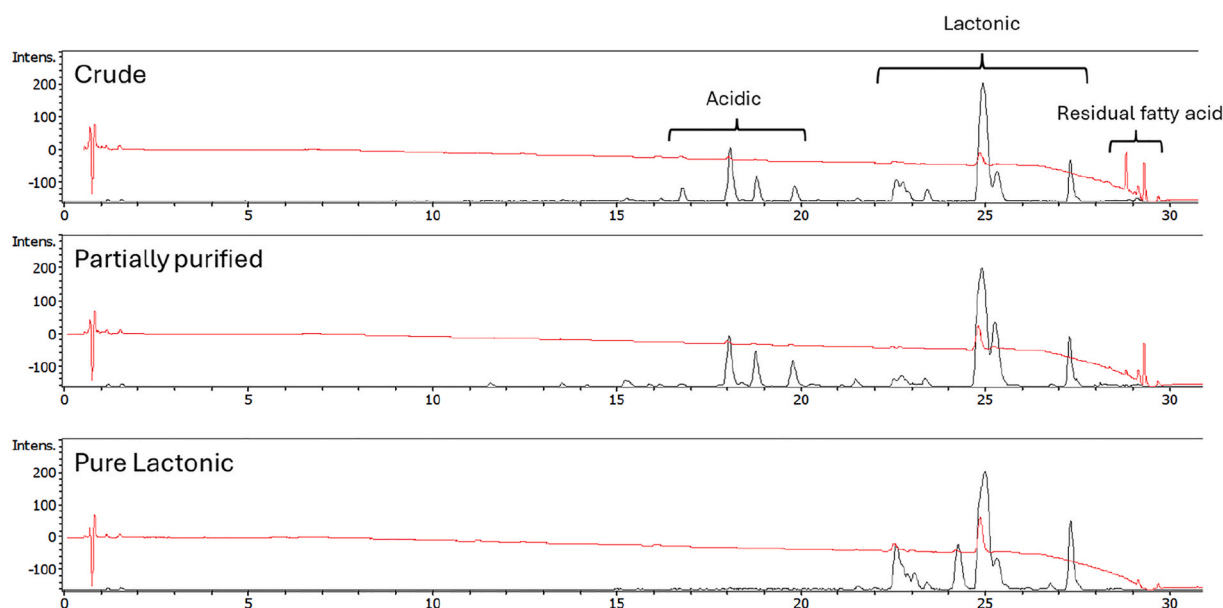


Fig. 1. Chromatograms for crude, partially purified, and purified SL samples. In red UV absorbance at 198 nm is shown, while in black MS spectra is shown. The main SL congener diacetylated C18:1 appears at min 25. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

purified SLs, enriched in lactonic C18:1 SL with decreased residual fatty acid content; and lastly iii) purified lactonic SLs, composed almost exclusively of lactonic diacetylated C18 SL congeners. These samples enabled the investigation into the specific effects of lactonic SL and the influence of co-extracted fermentation impurities on SL performance.

3.2. Self-assembling behaviour of SLs mixtures in water

Tensiometric measurements together with fluorescence analyses were employed to investigate the self-assembling behaviour of SLs in water and to determine the critical micelle concentration (CMC). Surface tension vs temperature plots for crude SLs, partially purified SLs, and purified SLs are shown in Fig. 2A. A marked decrease in surface tension (down to 35–40 mN/m) was observed for all SL samples, indicating that all mixtures, independently of the grade of purity of SLs, can act as amphiphilic compounds. Specifically, in all plots, the experimental data have a noticeable breakpoint that can be recognized as the CMC of the surfactant mixture. Above this point, surface tension values remained almost constant or slightly decreased, suggesting that the air–water interface is completely saturated by SLs in the mixtures.

Fig. 2B illustrates the reduction in fluorescence emission from the I/III pyrene peaks across all tested SL concentrations. This decrease suggests a shift in the microenvironment surrounding the pyrene probe, becoming increasingly hydrophobic due to its interaction with SL supramolecular aggregates. All profiles appear as well-shaped sigmoids from which CMC values can be calculated.

The CMC values calculated by the two techniques as well as the γ CMC from tensiometric analysis are reported in Table 1. A marked decrease in CMC values was observed moving from crude to partially purified and purified SLs (from ~ 140 μ g/mL to ~ 14 μ g/mL), while γ CMC were similar and in the range of 33–40 mN/m. The same trend of CMC values was also found from fluorescence analysis.

3.3. Characterization of the hybrid vesicles formed by SLs and phospholipids

Hybrid systems composed of phospholipids and SLs were prepared at different weight ratios (1:0.2, 1:0.6 and 1:1). After the preparation and extrusion, all samples provided aqueous dispersions in the nanometric range. Specifically, all the measured mean particle size after the preparation were in the range of 100–200 nm in terms of Z-average. The polydispersity of the population in terms of PDI was between 0.1 and 0.2 (with the only exception for samples DMPC:crSL 1:1 and DMPC:ppSL 1:1 having PDI between 0.2 and 0.3, where crSL stands for crude and ppSL stands for partially purified). Differences among samples were highlighted as a function of the storage time up to 63 days. No variations in Z-average and PDI were observed for systems prepared with purified SL (DMPC:prSL), while a progressive increase of both parameters was measured for all the other systems (DMPC:ppSL and DMPC:crSL). This

Table 1

Surface parameters for crude, partially purified, and purified SLs from tensiometric and fluorescence analyses at 25 °C.

	Tensiometry		Fluorescence
	CMC (μ g/mL)	γ CMC (mN/m)	CMC (μ g/mL)
Crude SL	140.0 $\pm$ 10.35	33.85 $\pm$ 1.23	116.3 $\pm$ 8.35
Partially purified SL	66.22 $\pm$ 4.23	36.15 $\pm$ 1.34	65.92 $\pm$ 3.24
Purified SL	14.03 $\pm$ 3.01	40.33 $\pm$ 1.29	44.56 $\pm$ 2.34

increase was more pronounced for the systems prepared at a DMPC:SL ratio of 1:1. Indeed, instability was observed and particle size above 1000 nm was measured for the sample DMPC:crSL at the ratio of 1:1 (Fig. 3). These results suggest that both the degree of SL purification and the relative weight ratio between phospholipids and SLs can affect the stability of the hybrid DMPC-SLs vesicles in terms of Z-average and PDI values. This is evident since more stable vesicles seem to be formed with purified SLs at all investigated DMPC:SL weight ratios and for crude or partially purified SLs but only at high DMPC:SL weight ratios (1:0.2 and 1:0.6).

To further investigate the possible incorporation of SLs in the liposomal membrane, HR-US analysis was performed (Fig. 4). This technique uses ultrasound waves at specific frequencies to monitor temperature-induced changes in the physical properties of the material. Variations in ultrasound parameters, such as sound speed and attenuation, are tracked over temperature. Thermal transitions are identified by deviations from the baseline in attenuation values, and by stepwise shifts interrupting the linear decrease in sound speed. By analysing sound speed profiles over temperature, clear stepwise shifts can be observed in the traces of pure DMPC (DMPC:SL 1:0) and samples prepared at DMPC:SL ratios of 1:0.2 and 1:0.6, referring to the sol-gel transition of liposomal bilayer. For samples prepared at the DMPC:SL ratio of 1:1, the transition is visible only when purified SL was employed. In the other cases (crude or partially purified SL), slightly decreasing or flat profiles were recorded, indicating that no thermal transition occurred in the analysed temperature range. Notably, flat profiles were also obtained from the analysis of SL dispersions without phospholipids (DMPC:SL 0:1), suggesting the absence of a thermal transition at least in the temperature range investigated (5–50 °C).

The calculated temperature related to the sol-gel transitions are reported in the supplementary table (Table ST). As well-known, the transition temperature of pure DMPC is ~ 24 °C. This temperature moved to slightly lower values in the presence of SLs, suggesting a possible incorporation or the interaction of SLs with the phospholipid of the liposomal membrane, at least for the DMPC:SL ratio of 1:0.2 and 1:0.6 for crude and partially purified SL and for the DMPC:SL ratio of 1:0.2, 1:0.6 and 1:1 for purified SL.

According to the obtained results regarding physical stability from DLS measurements and the HR-US analyses suggesting the possible

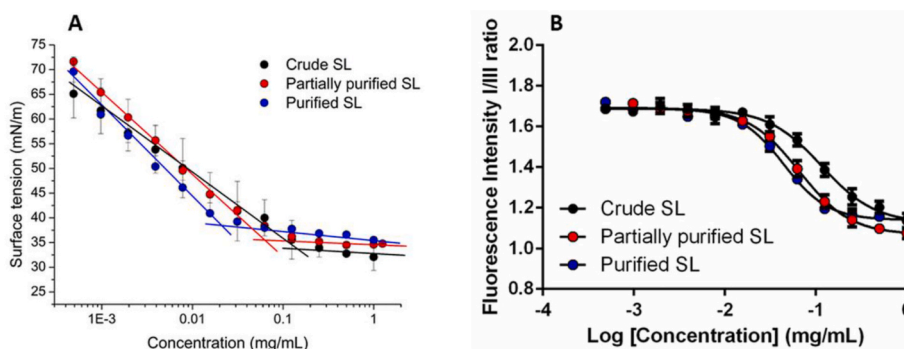


Fig. 2. Surface tension vs concentration plots for Crude SL, partially purified SL, and Purified SL (A); Fluorescence intensity (peak I/III) vs concentration plots for Crude SL, partially purified SL and Purified SL (B). Data are presented as mean $\pm$ SD (n = 3).

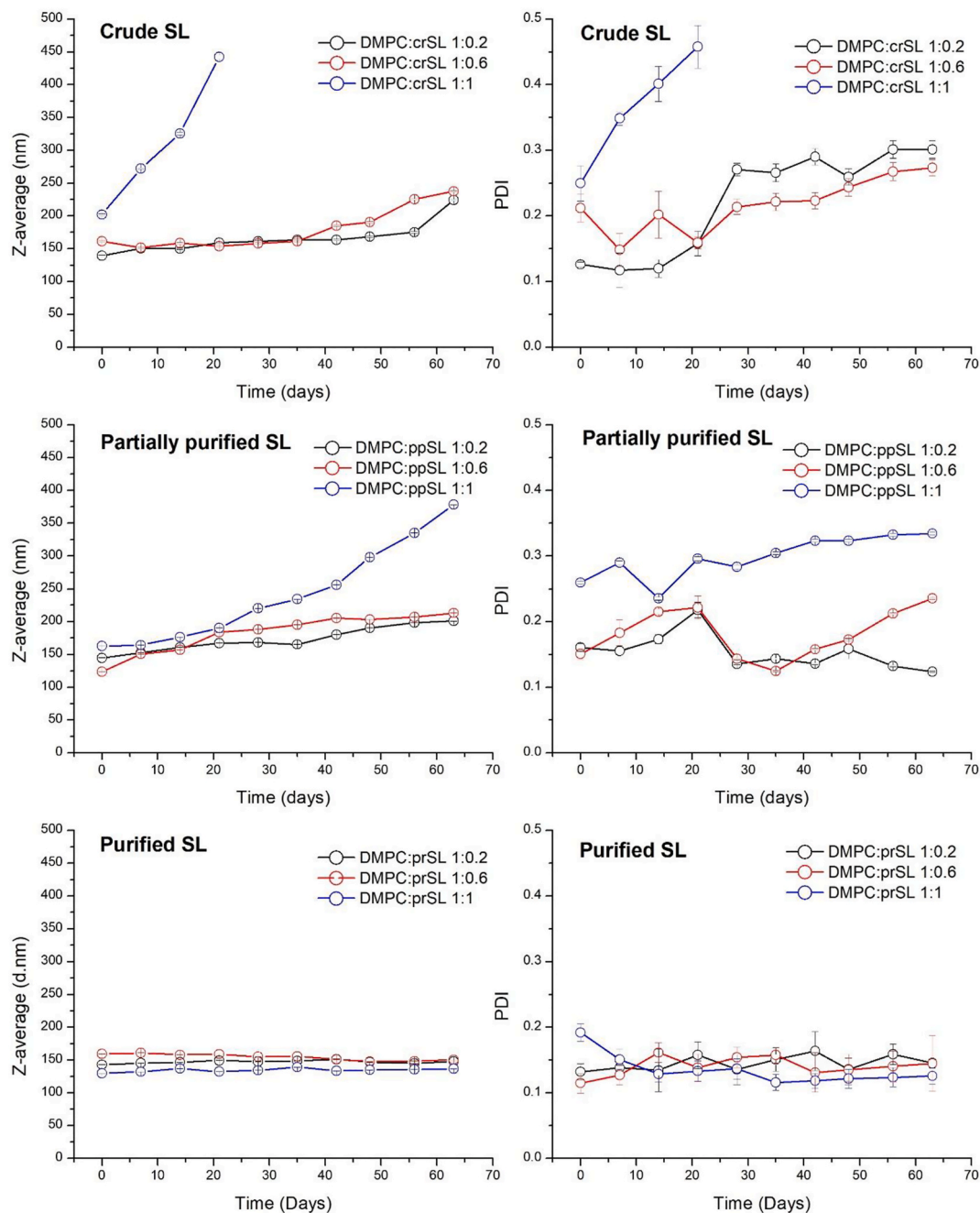


Fig. 3. Variation of Z-average (diameter, nm) and polydispersity index (PDI) over time (up to 63 days) for 5 mg/mL DMPC liposomal dispersions containing SLs (crude, CrSL; partially purified, ppSL; and purified, prSL) at different weight ratios (1:0.2, 1:0.6, 1:1). Data are presented as the mean $\pm$ SD (n = 3).

incorporation of SLs into phospholipid bilayer, the formulation DMPC: SL 1:0.6 (formed by crude, partially purified or purified SLs) was selected for further investigation.

3.4. Cryo-TEM analysis

The samples containing crude, partially purified, and purified SLs at the ratio 1:0.6 (DMPC: SL 1:0.6) were observed through Cryo-TEM (Fig. 5). The analysis confirmed the formation of generally unilamellar and spherical vesicular systems for all samples. From these pictures, volume % distributions of Feret diameter were built up and the median and the interquartile range (IQR) values calculated. The median values were 106.2, 152.0, and 138.6 nm for crude, partially purified and purified SL liposomal samples, respectively. These values were comparable

or slightly lower than those obtained from DLS analysis, according to the Intensity % distribution. The calculated IQR values were 35.2, 67.8, and 60.3 nm, for crude, partially purified, and purified liposomal samples, respectively, denoting a slightly narrower size distribution for liposomal dispersion containing crude SL than the other ones, at least according to the volume % distribution.

3.5. Antibacterial and antifungal activity of the hybrid vesicles formed by SLs and phospholipids

The antibacterial activity of crude, partially purified, and purified lactic SL was evaluated against a range of microbial strains, including both Gram-positive and Gram-negative bacteria and some *Candida* strains, belonging both to the ATCC collection and deriving from clinical

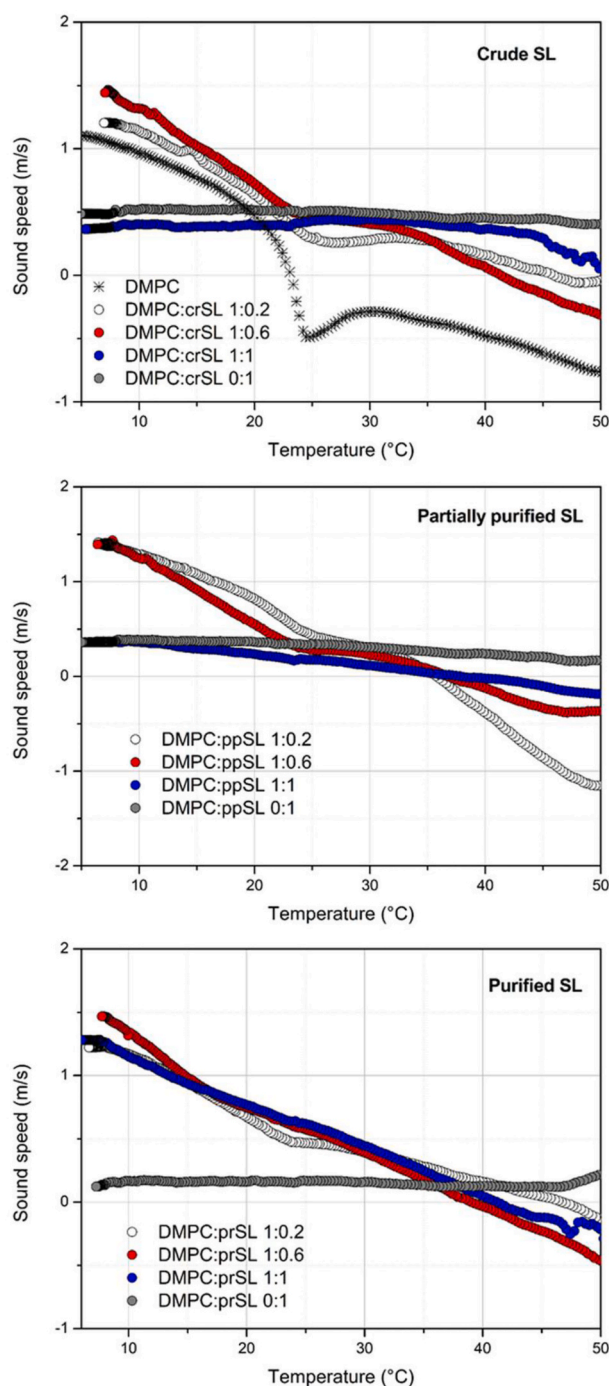


Fig. 4. HR-US signals (sound speed vs temperature) for the liposomal dispersions prepared with 5 mg/mL DMPC and SLs (crude, crSL; partially purified, ppSL; and purified, prSLpr) at different weight ratios (1:0.2, 1:0.6, 1:1) in comparison to 5 mg/mL DMPC and 5 mg/mL SLs (DMPC:SL 0:1).

isolation, using gentamicin or amphotericin B as reference drugs. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) values were determined for each bacterial strain to assess the efficacy of lactonic SLs and these values are reported in Table 2. A clear relationship between the purified lactonic SLs and their antimicrobial activity was highlighted. Indeed, as the purity of SLs increases, an enhancement in antimicrobial activity is observed, as indicated by the lowest MIC and MBC values calculated for the pure SLs with the respect to the crude and partially purified ones. This result highlights the relevancy of the purification procedure from a fermentation process

for SLs, being the calculated MIC and MBC values for the purified ones much more than two-fold lower than the others. Specifically, the purified SLs exhibited the most potent antibacterial activity against Gram-positive bacteria such as *Bacillus cereus* ATCC 10876, *Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecalis* BS, and *Staphylococcus aureus* BS with MIC values of 8 $\mu\text{g/mL}$. On the other hand, most of the gram-negative bacteria, such as *Escherichia coli* and *Klebsiella pneumoniae*, showed higher MIC values of 1024 $\mu\text{g/mL}$, indicating a lower susceptibility to purified SL compared to Gram-positive bacteria. Unexpectedly, a lower MIC value (16 $\mu\text{g/mL}$) was determined against the *Pseudomonas aeruginosa* strain, despite this Gram-negative bacterium usually shows resistance to many antimicrobial agents. This remarkable effect may be attributed to the unique surface-active and amphiphilic properties of SLs, which could disrupt the outer membrane of *P. aeruginosa*, causing “burst” holes in the cells, thereby enhancing permeability and leading to increased susceptibility [36,37]. For the partially purified SL, the MIC range against Gram-positive bacteria was in the range of 64–128 $\mu\text{g/mL}$, while many Gram-negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*) showed resistance. Exceptions included *Acinetobacter baumannii* with a MIC of 256 $\mu\text{g/mL}$ and *Pseudomonas aeruginosa* with a MIC of 128 $\mu\text{g/mL}$. The crude SLs exhibited even lower activity, with MIC values ranging between 256 and 512 $\mu\text{g/mL}$.

On the other side, a mild antifungal activity was observed against the tested *Candida* strains, as the purified SLs displayed a MIC value of 1024 $\mu\text{g/mL}$ and partially purified or crude SLs even higher MIC values (2048 $\mu\text{g/mL}$) or resistance.

Notably, especially for Gram-positive bacteria, the purified SL managed to match, and in some cases even exceed (for *Enterococcus faecalis* and *Staphylococcus aureus* BS), the action of gentamicin, which was used as the reference drug in our tests, confirming the remarkable antibacterial activity of these natural products. As regards the antifungal effect against *Candida* spp., the efficacy of SLs was much lower (>9-fold dilutions) than that of amphotericin B, used as reference drug.

Interestingly, the effect of SLs can be considered as bactericidal since the MIC and MBC values either corresponded or differed by only a single dilution ($\text{MBC/MIC} \leq 2$). Such a similarity between MIC and MBC values indicates that SLs may exert potent bactericidal effects [38].

Some of the Gram-positive, Gram-negative bacteria and yeast strains were chosen to assess the antimicrobial activity of liposomal formulations incorporating crude, partially purified, and purified SL (DMPC:SL 1:0.6 ratio). The MIC values of the samples normalised for the amount of SLs in the formulation in comparison to control are reported in Table 3. Control sample (without SLs) showed no activity towards the analysed species, while all liposomal formulations showed normalised MIC values comparable to those of the corresponding SLs. This result suggests that the antimicrobial activity, regardless the degree of purification, is retained by the DMPC-SLs hybrid vesicles in comparison to free SLs, at least *in vitro*.

3.6. Antiproliferative assay

The antiproliferative activity of crude, partially purified and purified SLs together with that of the vesicle formulation DMPC:prSL 1:0.6 against two breast cancer cell lines (MDA-MB-231 and SK-BR-3) is reported in Fig. 6 and Figure SF. All SLs, independently of the purity, showed a decrease in cell viability (%) on both cell lines (Figure SF). This decrease was more pronounced for the purified SLs since the calculated IC_{50} values moved from $\sim 89 \mu\text{g/mL}$ to $\sim 29 \mu\text{g/mL}$ for SK-BR-3 cell line and from $\sim 72 \mu\text{g/mL}$ to $\sim 19 \mu\text{g/mL}$ for MDA-MB-231 cell line. The antiproliferative activity of purified SLs, once formulating into hybrid phospholipid vesicles has been maintained on both cell line since the calculated IC_{50} values, after normalization for the amount of SLs in the formulation were similar to those of unformulated purified SLs ($\sim 19 \mu\text{g/mL}$ for SK-BR-3 cell line and $\sim 28 \mu\text{g/mL}$ for MDA-MB-231). The IC_{50} values for the vesicles composed of DMPC without SLs (negative control)

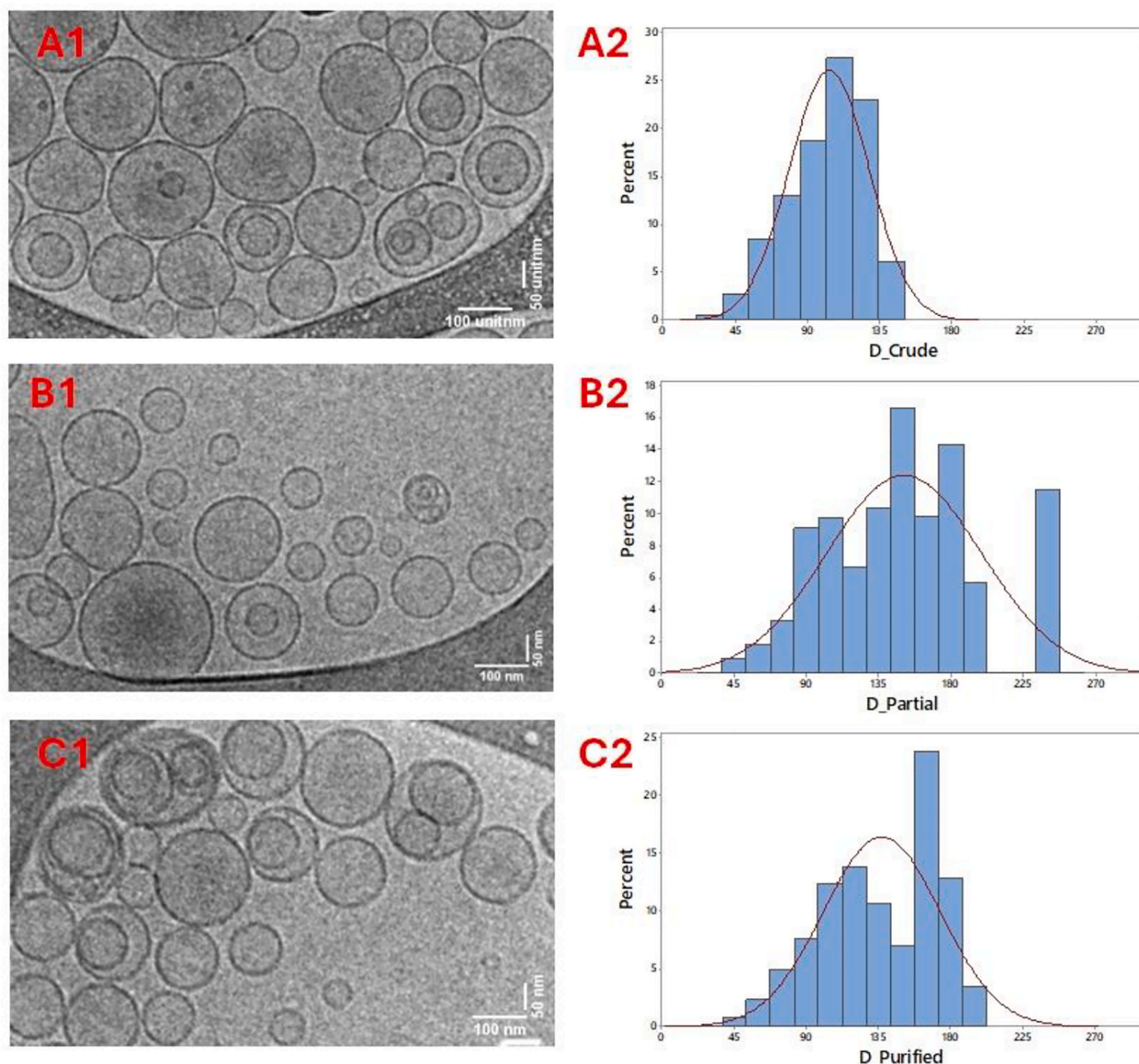


Fig. 5. Cryo-TEM images for DMPC:SL 1:0.6 (crude, partially purified, and purified, A1, B1, and C1, respectively) and relative particle size distribution (volume % distributions of Feret diameter; A2, B2 and C2, respectively).

were higher than 200 $\mu\text{g/mL}$ for both cell lines.

4. Discussion

SLs are glycolipid biosurfactants produced mainly by non-pathogenic yeasts such as *Starmerella bombicola*. Over the past years, they have attracted considerable attention due to their biocompatibility, biodegradability, and wide spectrum of biological activities, including antimicrobial, anticancer, and immunomodulatory effects [13,39,40]. Indeed, their functional properties are strongly influenced by their degree of purity and the formulation strategies applied for their delivery. For these reasons, the present article investigates how different purification levels (crude, partially purified, and purified) of SLs affect their antimicrobial and antiproliferative activities and further examines whether the possible incorporation into hybrid phospholipid-based vesicles can enhance their stability and bioactivity. In this work, SLs

mixtures were produced, with a different content of C18 lactonic diacetylated congeners and impurities. Specifically, crude SL was a mixture of acidic and lactonic SLs with high content of long chain fatty acids and $\sim 30\%$ C18:1; partially purified was essentially the same mixture of acidic and lactonic but with a reduced content of impurities and $\sim 60\%$ C18:1; purified SLs contain only lactonic diacetylated SLs with $\sim 90\%$ C18:1. All these mixtures showed a pronounced air-water surface activity in line with data reported in the literature. Indeed, Li et al. reported a CMC value of 15.0 mg/L and a γ_{CMC} value of 34.20 mN/m for lactonic SLs, which are superimposable to those calculated in the present study [41]. Similar values were also calculated by C18:0 diacetylated lactonic SLs (35.7 mN/m at CMC 29.2 mg/L) [42]. Thanks to the favourable biological properties of SLs, the development of novel formulations, including nanosystems, for SLs is desirable. One option is the formation of hybrid phospholipid-SLs vesicular systems. Specifically, the physical incorporation of SLs in phospholipid vesicles can modulate

Table 2

Antibacterial activity of purified SL, partially purified SL and crude SL (MIC, µg/mL).

Bacterial strains	Purified SL		Partially purified SL		Crude SL		Gentamicin
	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)
Gram-positive							
<i>Bacillus cereus</i> ATCC 10876	8	16	128	256	256	512	2
<i>Bacillus subtilis</i> ATCC 6633	8	16	64	128	256	512	0.5
<i>Enterococcus faecalis</i> ATCC 29212	8	8	64	128	256	512	16
<i>Enterococcus faecalis</i> BS	8	16	128	256	256	512	32
<i>Staphylococcus aureus</i> ATCC 6538p	16	32	128	256	266	512	2
<i>Staphylococcus aureus</i> ATCC 25923	32	32	128	256	256	512	0.5
<i>Staphylococcus aureus</i> ATCC 29213	16	16	128	256	256	512	0.5
<i>Staphylococcus aureus</i> ATCC 43300	32	32	128	256	256	512	16
<i>Staphylococcus aureus</i> BS	8	8	64	128	256	512	16
Gram-negative							
<i>Acinetobacter baumannii</i> ATCC 19606	64	128	256	512	512	1024	8
<i>Escherichia coli</i> ATCC 25922	1024	2048	R	R	R	R	2
<i>Escherichia coli</i> BS (ESBL)	1024	2048	R	R	R	R	32
<i>Klebsiella pneumoniae</i> ATCC 13883	1024	2048	R	R	R	R	16
<i>Klebsiella pneumoniae</i> BS	1024	2048	R	R	R	R	32
<i>Pseudomonas aeruginosa</i> ATCC 27853	16	16	128	256	256	512	2
Yeast strains							
<i>Candida albicans</i> ATCC 10231	1024	2048	2048	>2048	R	R	2
<i>Candida albicans</i> ATCC 90028	1024	2048	2048	>2048	R	R	1
<i>Candida krusei</i> ATCC 6258	1024	2048	2048	>2048	R	R	2
<i>Candida glabrata</i> ATCC 15126	1024	2048	2048	>2048	R	R	2

Table 3

Antibacterial activity of liposomal formulations prepared using purified SL, partially purified SL, and crude SL (MIC, µg/mL).

Bacterial strains	DMPC: prSL 1:0.6	DMPC: ppSL 1:0.6	DMPC:crSL 1:0.6	Control
	MIC (µg/ mL)	MIC (µg/ mL)	MIC (µg/ mL)	MIC (µg/ mL)
Gram-positive				
<i>Enterococcus faecalis</i> ATCC 29212	8	64	256	R
<i>Staphylococcus aureus</i> ATCC 29213	8	128	256	R
Gram-negative				
<i>Escherichia coli</i> ATCC 25922	1024	1024	R	
<i>Pseudomonas aeruginosa</i> ATCC 27853	16	128	256	
Yeast strains				
<i>Candida albicans</i> ATCC 10231	1024	2048	R	
<i>Candida albicans</i> ATCC 90028	1024	2048	R	

packing, phase behaviour, and domain formation, which can be harnessed to adjust vesicle leakage, fusion, or responsiveness, useful for drug delivery or other applications [25]. Moreover, phospholipid-based platforms are clinically validated and compatible with many routes of administration; adding SLs to these systems can provide eco-friendly vesicles to improve stability, circulation behaviour, or targeting SLs, therefore enhancing the antimicrobial or antiproliferative effects, by potentially reducing the required antibiotic/antifungal doses or enabling combination mechanisms [43,44]. Finally, embedding SLs inside phospholipid-based vesicles can be a strategy to increase their apparent solubility in water. It is known that lactonic SLs are water-poorly soluble, while acidic SLs are more hydrophilic and water-soluble than lactonic SLs, although the solubility of SLs increases with pH [13,45].

Despite solubility in pure water of crude, partially purified and

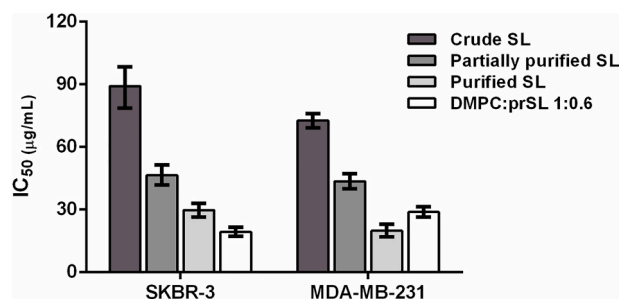


Fig. 6. The inhibitory concentration causing 50% of cells death (IC_{50} , µg/mL) for crude, partially purified, and purified SLs as determined from MTT assay on MDA-MB-231 and SK-BR-3 breast cancer cell lines in comparison to that of the liposomal formulation DMPC:prSL 1:0.6. The concentration is normalised for the amount of pure SLs in the liposomal formulation. The error bars define the 95% confidence interval as determined from dose-response curve non-linear regression.

purified SLs was not analytically determined, samples prepared at a concentration above ~0.5 mg/mL for crude and partially purified SLs and samples at a concentration above 1 mg/mL for purified SLs were slightly cloudy, indicating that the solubility has been reached. The addition of crude, partially purified and purified SLs to phospholipid dispersion in water to form hybrid phospholipid-based vesicles has improved the apparent solubility of SLs, as suggested by HR-US results, for which typical profiles of hybrid vesicles were recorded, at least for DMPC:SL 1:0.2 and DMPC:SL 1:0.6 samples [46]. In this regards, our results are in line with those from a previous study reporting that lactonic SLs (purity >85%) stabilized and homogenized liposomal vesicles as resulted from lowered Z-average, PDI and zeta potential values [26].

The incorporation of SLs in phospholipid bilayers is a crucial aspect for the development of hybrid nanovesicles. However, few studies have been conducted so far to investigate the interaction between SLs and phospholipids [25,47] and the mechanism underlying these interactions still remains not completely elucidated. Acidic SLs were found to interact with DPPC acyl chain and polar headgroup regions, promoting

chain ordering and greater hydration of the membrane interface. Moreover, differential scanning calorimetry (DSC) revealed that, even at low molar fractions, SLs modified the phase behaviour of DPPC, inducing fluid–fluid phase separation and promoting the formation of biosurfactant-enriched domains within the bilayer, as resulted from the decrease in the transition temperature and enthalpy of lipid bilayer as a function of the SL content [25]. On the other side, studies from the literature have also shown that SLs can form by themselves vesicles, membranes or other supramolecular aggregates in aqueous environment as a function of temperature and concentration [48,49]. Moreover, their interactions with lipid bilayers as well as those of other glycolipid surfactants, can be quite complex leading to partial incorporation (vesicle-in-vesicle aggregates, inner tubulation, vesosomes formation) or to a complete portioning inside the phospholipid bilayers causing the formation of enriched microdomains, altered packing or vesicle shape deformations (such as budding and invagination) [50]. Despite a comprehensive investigation on the interaction between SLs and DMPC bilayers to form hybrid liposomes has not been carried out in our work, the preliminary results from DLS, HR-US and Cryo-TEM seem to confirm those reported for acid SLs and DPPC bilayers [25]. Indeed, the lactonic SLs (as a function of their purification grade) were able to modify the thermal phase behaviour of DMPC bilayers at a different extent, as resulted from the broadening of sound speed profiles and the decrease of the calculated main transition temperature, suggesting at least a partial portioning of SLs inside DMPC bilayers.

Another relevant aspect is the effect of the level of purity of SLs in terms of hybrid vesicles formation and stability, which has not been extensively investigated in the literature. Our results demonstrated that a longer stability of the hybrid vesicles over time was achieved for pure lactonic SLs, since they could be better portioned inside the phospholipid membrane. The heterogeneity in composition for partially purified and crude SLs could lead to inconsistent packing, bilayer defects, thereby destabilizing vesicles and accelerating leakage or aggregation [25]. Consistently with the work from Chen and Zhang [26], the hybrid systems prepared up to a weight ratio phospholipid to SLs 1:1 (DMPC:SL 1:1) remain stable over time in terms of Z-average and PDI. Additional investigations using confocal microscopy, small angle scattering techniques and nuclear magnetic resonance would be helpful to provide more structural insights. On these premises, mixing phospholipids and SLs could indeed involve the formation of a bicomponent membrane, but mixed vesicles composed of incorporated SLs into phospholipid-based structures can also be considered. However, our preliminary results on the interaction between lactonic SLs and phospholipids are encouraging for the development of hybrid nanosystems, which could be employed in different areas to enhance the SLs bioactivity.

As such, the antiproliferative effect of lactonic SLs has been demonstrated on different cancer cell lines, including breast, lung, liver, cervical, and colon, by acting through several mechanisms related to apoptosis, such as caspase activation, upregulation or downregulation of the B-cell lymphoma (Bcl)-family proteins, and alteration in mitochondrial membrane permeability [39]. As regards breast cancer cell lines, the obtained cytotoxicity results for SLs confirmed those from the literature [42,51]. Ribeiro et al. found that IC₅₀ values against DA-MB-231 cells for C18:0 diacetylate SL was 15 µg/mL, which is comparable to that found in our study (~19 µg/mL). As for the CMC, IC₅₀ values also decrease as a function of SLs purification, suggesting that surface properties are somehow involved into the cytotoxic effect, which is intrinsically related to the SLs concentration. On the other side, no cytotoxicity data are available for SLs against SK-BR-3 breast cell line, which resulted to be slightly less susceptible than DA-MB-231.

As regards the antimicrobial activity, especially pure SLs display a high antimicrobial effect, superior to many of the natural products. Therefore, SLs could be hopefully employed for the development of effective and innovative solutions to combat infections and antibiotic resistance. Specifically, the relevant influence of lactonic SLs and their

degree of purity on antimicrobial efficacy against Gram-positive, Gram-negative bacteria, and fungal species have been highlighted. Generally, lactonic SLs display a more potent antimicrobial activity than the corresponding acidic SLs, thanks to their higher lipophilicity and superior ability to disrupt cell membranes [52,53]. The tested lactonic SLs exhibited consistently greater antimicrobial activity against Gram-positive strains rather than against Gram-negative bacteria, aligning with previous results [37,54]. Specifically, the reported MIC values of purified lactonic C18 SLs against *Staphylococcus aureus* are in the range of 31.25–50 µg/mL, which are in line or slightly higher than those reported in our study [37,55]. As regards Gram-negative bacteria such as *Escherichia coli*, MIC values higher than 1000 µg/mL can be found [38]. Studies in the literature indicate that SLs exert their antimicrobial effects primarily through membrane disruption, thereby acting as bactericidal agents against both Gram-positive and Gram-negative bacteria (despite the different susceptibility) as confirmed by our results (MBC/MIC ≤ 2) [18,37,38]. Their role as bactericidal agents supports them as attractive candidates for the development of complementary or combined therapies in infectious disease management.

SLs purification emerges as a critical aspect to be considered in terms of SLs bioactivity. Indeed, both the composition (acidic vs lactonic), the content of SLs preparations (crude or purified) or chemical modification can relevantly affect their antimicrobial activity [56,57]. Generally, purified lactonic SLs exhibit higher antibacterial activity than the acidic forms or crude mixtures [58,59]. Some studies have investigated the effect of SL purification regarding the antimicrobial activity, highlighting the superior effect of the lactonic purified fractions (≥95% content) over raw or semi-purified mixtures, albeit a lower activity generally is maintained especially against Gram-positive bacteria as *S. aureus* [58,60]. However, a definitive explanation regarding the more potent activity and more reliable and reproducible results for purified fractions has not been provided since several contributions can be involved. For instance, the presence of impurities and by-products can interfere with the direct interaction between SLs and the bacterial cell membrane. As purity increases, SLs can more efficiently penetrate and destabilize bacterial membranes, leading to an improved bactericidal effect [61]. Higher-purity SLs could also facilitate stronger binding of functional groups to bacterial cells, thereby enhancing their bioactivity [44]. On the other side, the purification and fractionation process could increase the concentration of the active compounds (mainly lactonic SLs) over inactive or less active constituents of the mixtures (such as sugars or free fatty acids), ensuring more efficient interactions at lower inhibitory concentrations [58]. Interestingly, the *in vitro* antiproliferative and antimicrobial activities of hybrid DMPC-SLs nanosystems have been maintained in comparison to that of free SLs, thus encouraging the continuous research in the field of hybrid phospholipid-SLs nanovesicles to enhance the bioavailability and activity *in vivo*.

5. Conclusions

The study highlighted the feasibility of preparing lactonic SLs (mainly diacetylated lactonic C18:1), produced from waste through a solid-state fermentation process, into hybrid phospholipid-based vesicles. The formation of hybrid SLs-DMPC nanovesicles can represent a strategy to improve the administration and therapeutic applications of SLs. The physicochemical properties, such as CMC values of SLs mixtures as well as the particles size and the physical stability of the resulting nanovesicles are markedly influenced by SLs degree of purity. Indeed, lower CMC values and improved vesicle stability were achieved for purified SLs. Furthermore, the purification strongly enhanced the antimicrobial and antiproliferative activities. Indeed, purified SLs displayed an antibacterial activity against Gram-positive with MIC values comparable to the reference drugs, gentamicin, while lower activity was observed against selected Gram-negative strains and *Candida* species. In

addition, the antiproliferative assay revealed marked cytotoxic effects of purified SLs against selected breast cancer cell lines ((MDA-MB-231 and SK-BR-3). The formation of hybrid nanovesicles composed of SLs and DMPC maintained or even improved the biological activities (antimicrobial or antiproliferative), highlighting the potential of these hybrid nanostructures as delivery platforms for SLs. These findings confirmed the role of purification in maximizing the bioactivity of SLs and provide a foundation to fully exploit the potential of these natural glycolipids in antimicrobial and anticancer therapies.

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CRediT authorship contribution statement

Diego Romano Perinelli: Conceptualization, Investigation, Methodology, Project administration, Writing – original draft. **Teresa Gea:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Alexia Barbarossa:** Investigation, Methodology, Writing – review & editing. **Junbiao Wang:** Investigation, Methodology. **Marco Cespi:** Data curation, Formal analysis. **Nicolás Oiza:** Investigation, Methodology. **Xavier Font:** Supervision, Writing – review & editing. **Alessia Carocci:** Methodology, Supervision. **Antonio Rosato:** Supervision, Visualization. **Eleonora Spinuzzi:** Visualization. **Cristina Marchini:** Supervision. **Giulia Bonacucina:** Supervision, Writing – review & editing.

Declaration of competing interest

Teresa Gea reports financial support was provided by Spanish Ministerio de Ciencia, Innovación y Universidades. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jddst.2026.108282>.

Data availability

Data will be made available on request.

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