Green catanionic gemini surfactant–lichenysin mixture: improved surface, antimicrobial and physiological properties

Albert Ruiz1,2, Aurora Pinazo1, Lourdes Pérez1, Angels Manresa2, Ana M. Marqués2*

1Department of Chemical and Surfactant Technology, IQAC-CSIC, Barcelona 08034, Spain
2Lab. of Microbiology, Dept. of Biology, Health and Environment, Fac. of Pharmacy and Food Science, University of Barcelona, Barcelona 08028, Spain

ABSTRACT: Catanionic surfactant mixtures form a wide variety of organized assemblies and aggregates with improved physicochemical and biological properties. The green catanionic mixture C₃(CA)₂:lichenysin (molar ratio 8:2) showed antimicrobial synergies against Yersinia enterocolitica, Bacillus subtilis, Escherichia coli O157:H7 and Candida albicans. Flow cytometry and viability studies indicated that this catanionic mixture increases the probability of Y. enterocolitica (38.2%) and B. subtilis (17.1%) cells entering a viable but nonculturable state. Zeta potential showed that one of the cationic charges of C₃(CA)₂ is neutralised by lichenysin. An isotherm study demonstrated the formation of a stable aggregate between the two surfactants that was able to interact with bacterial phospholipids. The lowest hemolysis (22.1μM) was obtained with the catanionic mixture, although an irritant potential (0.70) was characterised. According to the therapeutic index, the C₃(CA)₂:lichenysin mixture was the formulation least toxic to eukaryotic cells. Partial neutralisation of C₃(CA)₂ by lichenysin modified the mode of action that enhances the transition of bacterial cells into a viable but nonculturable state (VBNC) and improved the cell selectivity.
INTRODUCTION

Surface active molecules or surfactants are an extensive group of amphiphilic compounds bearing both a hydrophilic head group and a hydrophobic hydrocarbon chain, which allows them to interact at the interfaces between aqueous and non-aqueous systems, thus reducing the surface tension. The properties and efficiency of a surfactant are determined by the relative size of its hydrophobic and hydrophilic parts, the presence of charges, and degree of hydration. The hydrophobic end consists of a saturated or unsaturated alkyl chain of different lengths and flexibility. The hydrophilic head group can be cationic (positively charged), anionic (negatively charged), zwitterion (neutral with charges), or non-ionic (without charge). In an aqueous environment, when surfactants saturate the interface, they form aggregates called micelles, which minimize the free energy of the solution. The lowest saturation concentration is known as the critical micellar concentration (CMC).

Surfactants are used for their surface activity as ingredients of many formulations, including household detergents and personal care products, and have extensive usage in the pharmaceutical, petrochemical and mining industries. They also have potential applications in hi-tech fields such as nanotechnology and molecular biology. Additionally, many studies have reported antimicrobial activity of cationic surfactants. Considering the global problem of antibiotic resistance, this opens the possibility of using surfactants to reduce antibiotic or preservative consumption, even though their antimicrobial properties are far from the level of antibiotics. A combination of surface and antimicrobial activity is also very interesting for industrial applications. Current research is seeking...
new surface active molecules to obtain novel surfactants that are more effective, environmentally friendly and with enhanced antimicrobial activity.\textsuperscript{3-5}

Amino acid-based surfactants are added-value products with a hydrophilic moiety based on one or more amino acids from enzymatic synthesis or protein hydrolysis, and a hydrophobic moiety obtained from natural oils. Their synthesis and properties meet most of the principles of Green Chemistry: prevention of waste, atom economy, the use of less hazardous chemicals, safe materials, and renewable feedstocks, catalysis and design of degradable products. They are a promising alternative to conventional synthetic surfactants due to their enhanced surface activity, antimicrobial properties, biodegradability and sustainable production.\textsuperscript{5}

Biosurfactants produced by microorganisms are sustainable products that could replace chemical synthesis-based surfactants. Among them, lipopeptides are produced by various bacteria and are composed of a $\beta$-hydroxyl fatty chain bound to a cyclic polypeptide.\textsuperscript{6} Lichenysin is a cyclic lipopeptide similar to surfactin synthesized by \textit{Bacillus licheniformis}.\textsuperscript{7} Coronel-León \textit{et al.}\textsuperscript{8} characterised the isoforms of lichenysin (Figure 1A) produced by \textit{B. licheniformis} AL1.1, isolated from Antarctic soil samples.\textsuperscript{9} Lichenysin\textsubscript{AL1.1} has a cyclic peptide bearing seven amino acids as a large polar head, including an anionic residue of L-Asp, and a $\beta$-hydroxyl fatty chain of 14 carbons, although chains of 15 and 16 carbons were also found. Thus, it is a surface-active molecule with a negative charge and a mean molecular weight of 1043 g/mol, which reduces the surface tension of water to 28.5 mN/m at the CMC of 14.4 $\mu$M.

Typically, mixtures of anionic and cationic surfactants in aqueous solutions contain catanionic vesicles. These aggregates have attracted attention due to their morphological similarity with classical liposomes, over which they have several advantages: they can be prepared using inexpensive materials and without the input of mechanical energy, and the resulting formulations
are thermodynamically stable for long periods of time. Recently it has been demonstrated that catanionic mixtures may also have improved biological properties, attributed to synergistic effects, compared with those of the individual components.

In a previous study, the catanionic mixture of lichenysin and an arginine-based surfactant showed synergistic antimicrobial activity. These results prompted us to explore new catanionic mixtures (lichenysin plus arginine-based surfactants) in order to define the molecular requirements in their chemical structures for synergistic antimicrobial activity, and to characterise their mode of action and physicochemical and physiological properties.

**EXPERIMENTAL SECTION**

**Materials.** $N^\alpha N^\omega$-bis($N^\alpha$caproylarginine) $\alpha, \omega$-propyldiamide ($C_3(CA)_2$) (Figure 1B) and $N^\alpha$-caproyl-arginine methyl ester (CAM) (Figure 1C) are arginine-based surfactants. CAM is a single-chain surfactant with 10 atoms in the fatty chain, while $C_3(CA)_2$ is a gemini surfactant arising from the dimerization of two molecules of CAM, connected by a spacer chain of 3 carbon atoms. $C_3(CA)_2$ and CAM have molecular weights of 766.5 and 378 g/mol, and reduce the surface tension of water to 32 and 40 mN/m at CMCs of 4.3 and 16 mM, respectively.
Figure 1. Chemical structures of lichenysin (A), C₃(CA)₂ (B) and CAM (C).

**Microorganisms and culture conditions.** Lichenysin was produced by *B. licheniformis* AL 1.1 in cultures of up to 100 mL of mineral medium (MM) (glucose 10 g/L, KH₂PO₄ 4 g/L, Na₂HPO₄ 5.7 g/L, (NH₄)₂HPO₄ 6.6 g/L, FeSO₄·4H₂O 0.01 g/L, MgSO₄·7H₂O 0.21 g/L, CaCl₂ 7·10⁻⁶ g/L and oligo-elements solution, 0.05 mL/L: H₃BO₃ 0.148 g, CuSO₄·5H₂O 0.196 g, MnSO₄·H₂O 0.154 g, Na₂MoO₄·2H₂O 0.15 g and ZnSO₄·7H₂O 0.307 g in 100 mL of oligo-elements solution) in baffled flasks, adapted from Coronel-León *et al.*⁸ The inoculum was a suspension equivalent to
McFarland standard n.4 of *B. licheniformis* AL1.1 prepared in Ringer’s solution (Scharlau Chemie, Spain) from overnight colonies grown on TSA plates (Conda Pronadisa, Spain). It was inoculated in a final concentration of 2% (v/v) and incubated for 72 hours at 30ºC in agitation at 120 rpm.

Strains used to test the antimicrobial activity were *Escherichia coli* ATCC 25922, *Escherichia coli* O157:H7 CECT 4267, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 13883, *Yersinia enterocolitica* ATCC 9610, *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* methicillin-resistant (MRSA) ATCC 43300, *Staphylococcus epidermis* ATCC 12228, *Bacillus subtilis* ATCC 6633, *Listeria monocytogenes* ATCC 15313, *Kocuria rhizophila* ATCC 9341 and *Candida albicans* ATCC 10231.

**Lichenysin recovery.** After 72 hours, the culture on MM was centrifuged (9000 rpm for 20 min at 4ºC) and the supernatant was lyophilised (Cryodos from Telstar) until complete dryness. Three consecutive organic liquid/liquid extractions with ethyl acetate:methanol 8:1 (V:V) were performed from the lyophilization product solubilized in up to 5 ml of water. The organic phase was filtered with Whatman n.1 filter paper. The solvent was evaporated in vacuum and the organic extract was recovered in the desired solvent or dried.

**Surface tension (γ) measurement.** The ring technique was used to estimate the surface tension of culture supernatants with a Tensiometer K9 (Krüss, Germany). The instrument was calibrated against ultrapure water (γ\text{ST}=72.3 \text{ mN/m}) and pure ethanol (γ\text{ST}=22.7 \text{ mN/m}). Measurements were performed in triplicate at a constant temperature (25ºC).

**Zeta-potential.** Lichenysin, C₃(CA)₂, CAM and mixtures of C₃(CA)₂:lichenysin, 5:5 and 8:2 (mol:mol), and CAM:lichenysin, 5:5 and 8:2 (mol:mol), were prepared in concentrations of 0.5 mM in miliQ water. The zeta-potential of the aggregates was obtained by analyzing the samples in a Zetasizer Malvern Nano-ZS using a ZeNO112 cell. The value was taken as the mean of three
independent measurements. Each measurement was in turn the average of ten sub-measurements of 20 s each.

**Monolayer isotherms.** Monolayer isotherms (surface pressure versus mean molecular area, $\pi$-A) at 37°C were measured using a Langmuir balance (KSV Instruments Minitrough, Finland) and a paper Wilhelmy plate (Whatman ashless) as detailed in Lozano et al.\textsuperscript{15} The surface pressure is defined as $\pi=\gamma_0-\gamma$, where $\gamma_0$ is the water surface tension (72.3 mN/m). Tris buffer 20 mM (Merck, Germany) at pH 6.8 was used as the subphase. Monolayers of lichenysin, C$_3$(CA)$_2$, CAM and an *E. coli* total lipid extract (TLE) (Avanti Polar Lipids, USA) were studied. Weight composition and charges at pH 6.8 of the TLE were phosphatidylethanolamine 57.5% (zwitterionic), phosphatidylglycerol 15.1% (one negative charge), cardiolipin 9.8% (two negative charges) and an unknown fraction 17.6%. The mean molecular weight was estimated as 772.75 g/mol from the phospholipid fraction. Aliquots of 25 $\mu$L of single components, catanionic mixtures of surfactants:lichenysin (molar ratio 8:2) and mixtures with TLE (volume ratio 8:2), all prepared in chloroform:ethanol 9:1 (V:V) at 1 mg/mL, were spread on the surface with a microsyringe (Hamilton 50±1 $\mu$L). The subphase was agitated with a stirrer and evaporation of the solvent was allowed for 15 min. The rate of symmetric compression was 20 mm/min. The surface pressure was monitored by the plate’s weight. Each isotherm was measured at least twice. The $\pi$-A curve was plotted using the statistical software *OriginPro 8*. Mixed isotherms were analyzed as binary mixtures of monolayers. The excess of free energy of the mixture ($\Delta G_m^{ex}$) was calculated considering $\Delta G_m^{ex} = \int_0^\infty (A_{12}-x_1*A_1-x_2*A_2)d\pi$ where $A_{12}$ is the mean molecular area of the binary mixture, $x_1$ and $x_2$ are the molar fractions of the components in the mixture and $A_1$ and $A_2$ are the respective molecular area of each component in a pure monolayer.\textsuperscript{16} Integrations were performed from inverted $\pi$-A curves using *OriginPro 8*. 
**Minimal inhibitory concentration (MIC) determination.** MIC was determined for lichenysin, C₃(CA)₂, CAM, and their mixtures at a molar ratio of 8:2 (surfactant:lichenysin) as detailed in Coronel-León et al.¹² Suspensions in Ringer’s solution from overnight cultures on TSA of the corresponding microorganism, *L. monocytogenes* cultured on Brain-heart infusion agar (ThermoFisher scientific, England), were used as the inoculum. Cell concentrations were estimated spectrophotometrically to obtain a final cell concentration in the well of 5·10⁵ cfu/mL of the tested bacteria, or 2.5·10³ cfu/mL of *C. albicans*. Plates were incubated for 16-20 h at 37ºC for bacteria and 46-50h for *C. albicans*. Synergy between the products was determined using the fractional inhibitory concentration (FIC) that is defined as:

\[ \text{FIC} = (a \times \frac{\text{MIC}_m}{\text{MIC}_a}) + (b \times \frac{\text{MIC}_m}{\text{MIC}_b}) \]

where a and b are the percentages of surfactant and lichenysin in the mixture, respectively, MICₘ is the MIC of the mixture, and MICₐ and MICₕ are the MICs of the surfactant and the lichenysin, respectively. Synergy was considered present only when FIC values were below 0.5.¹⁷

**Exposure of microorganisms to surfactants.** The inoculum was prepared as in the MIC determination. Contacts between microorganisms (*E. coli* O157:H7, *Y. enterocolitica*, *B. subtilis* and *C. albicans*) and products (C₃(CA)₂ and C₃(CA)₂:lichenysin 8:2 (mol:mol)) took place in buffered peptone water (BPW) (ThermoFisher scientific, England), supplemented with MIC values of the products and 10% (V/V) of inoculum: 10⁷ cfu/mL for bacteria and 2·10⁵ cfu/mL for *C. albicans*, and incubated at room temperature.

**Viability assay.** At time 0 and every 30 minutes, 1 mL of sample was taken and immediately diluted 1/10 in Ringer’s solution to stop the effect of the product. A cell count was performed for each sample by preparing serial dilutions, inoculating TSA plates with 100 µL inoculum and incubating for 16h at 37ºC.
**Flow cytometry (FC).** Negative and positive controls were performed in parallel, with incubations without products and with heat treatment (70°C), respectively. At 150 min for *Y. enterocolitica*, 105 min for *B. subtilis* and 90 min for *E. coli* O157:H7 and *C. albicans*, 10 mL of the samples were taken and centrifuged (9000 rpm for 20 min at 4°C). The pellet was suspended in 2 mL of BPW and diluted 1/100 in filtered PBS (pH 7.4) at a final volume of 500 μL. In parallel, a cell count was performed. All the samples were stained with 1 μL of bis-oxonol (BOX) (250 μM in ethanol), and 5 μL of propidium iodide (PI) (1 mg/mL in water), and incubated for 5 min at room temperature. 200 μL to 500 μL of sample were loaded in the FC Cytomics FC500NPL (Beckman Coulter, USA). Fluorescence from BOX (560 nm) and PI (617 nm) was detected.

**Transmission electronic microscopy (TEM).** Negative controls were performed in parallel with incubations without products. At the same times as the FC assay, all the suspension volume was centrifuged at 9000 rpm for 20 min at 4°C. The pellet was resuspended in 1 mL of BPW and centrifuged again at 10000 rpm for 10 minutes. The pellet was fixed with glutaraldehyde 2.5% with 0.1M phosphate buffer (PB) at 4°C for 2 hours. Samples were centrifuged (2500 rpm for 10 minutes), washed four times with 0.1M PB (4°C) and fixed with 0.1M PB with 1% of OsO₄ and 0.8% of K₄[Fe(CN)₆] (4°C for 2 hours in darkness). Four washes were performed with miliQ water and then by 0.1M PB. The sample was dehydrated at 4°C with sequential concentrated acetone: 50, 70, 90 and 96% (10 minutes), and 100% (15 minutes). Thereafter, samples were infiltrated, polymerized, sectioned and mounted as reported in Colomer *et al.* Ultrathin sections were observed in a JEOL 1010 microscope (EM). 80Kv images were acquired using a CCD Megaview 1kx1k.

**Red blood cell (RBC) assay and therapeutic index.** Hemolytic activity, denaturation of hemoglobin and irritancy potential of the corresponding concentration ranges of lichenysin (1-15
μM), C₃(CA)₂ (5-40 μM) and C₃(CA)₂:lichenysin 8:2 (mol:mol) (9-23 μM) were determined using a protocol adapted from Pape and Hoppe.²⁹ A rabbit blood sample, supplied by the Animal Experimentation Unit (Centres Científics I Tecnològis de la UB, CCiTUB), was cleaned thrice with Hepes buffer (150mM NaCl, 5mM Hepes, pH 7.4) on ice and diluted until reaching Abs₅₄₀nm=1. Eight dilutions below the CMC of pure surfactants and the catanionic mixture were prepared, concentrated 20-fold. 10 μL of the concentrated samples were added to 200 μL of erythrocyte solution (in triplicate), incubated for 20 min at 37ºC and centrifuged at 3000rpm for 4 minutes (Allegra™ 25R, Beckman Coulter). Supernatants were collected and analyzed by a spectrophotometer at 540 and 575 nm (Sinergy HT microplate reader, Biotek). The extent of hemolysis was determined by the relative proportion of absorbance at 540 nm of each sample against a completely hemolyzed control (erythrocyte solution with distilled water in the same conditions). The concentration at which hemolysis (H₅₀) occurred in 50% of red blood cells (RBC) was calculated from concentration-hemolysis curves. The denaturation index (DI) was calculated as DI=([R₁-R₂]/[R₁-R₃])*100, where R₁ is 1.05, the constant ratio Abs₅₇₅/Abs₅₄₀ of oxyhemoglobin, R₂ is the ratio Abs₅₇₅/Abs₅₄₀ of the hemolysis of 100 mg/mL of SDS, and R₃ is the ratio Abs₅₇₅/Abs₅₄₀ of the samples. Irritancy potential was calculated as the H₅₀ [μg/mL] / DI [%] quotient from the results after 20 minutes of incubation. A physiological correlation can be determined using the following scale: non-irritant (>100), slightly irritant (>10), moderately irritant (>1), irritant (>0.1) and very irritant (<0.1).²⁹ Relative selectivity for each microorganism strain against eukaryotic cells corresponding to the three surfactant formulations tested was calculated using the therapeutic index (TI), defined as the H₅₀/MIC quotient. The larger the TI is, the more selective against microorganisms, and so less toxic against eukaryotic cells.⁴

RESULTS AND DISCUSSION
**Production, extraction and characterization of lichenysin.** Lichenysin was produced in the culture supernatant, which after 72 hours of incubation had a surface tension of 30±2 mN/m. Lichenysin was recovered using the lyophilization-based concentration method, with a final productivity of crude extract up to 351.5 mg/L. The organic extract and purified lichenysin were compared by TLC to check purity. From here onwards the organic extract will be referred to as lichenysin.

**Zeta potential.** The zeta potential of C$_3$(CA)$_2$, CAM, lichenysin and its binary mixtures was measured to study how the surfactants interact in catanionic mixtures. The zeta potential of C$_3$(CA)$_2$ and CAM was 60 and 30 mV, respectively, while that of lichenysin was -30 mV. These values correspond to the known charge of each surfactant: two positive charges of C$_3$(CA)$_2$, one positive charge of CAM and one negative charge of lichenysin. When C$_3$(CA)$_2$ was mixed with lichenysin at a molar ratio of 5:5, the charge was neutralized to 38.1 mV, while the mixture CAM:lichenysin at the same molar ratio was neutralized to -2.8 mV, almost zero. This indicates that one positive charge of the surfactants interacted with one negative charge of lichenysin. The overall zeta potential of the mixtures of C$_3$(CA)$_2$ and CAM with lichenysin at 8:2 (mol:mol) presented a proportional neutralisation of the charge: 45.5 mV for C$_3$(CA)$_2$ and 26.8 mV for CAM.

**Isotherms (π-A) of mixed monolayers.** Understanding the behavior of mixed monolayers is of great interest because it provides insight into the interactions between the monolayer compounds and contributes to a general study model for biological systems. To study the interaction of lichenysin with the arginine-based surfactants, a simple monolayer membrane model was used. The aim of these experiments was to figure out the mechanism of the surfactant interaction with bacterial membranes. Thus, the behavior of monolayers of mixtures of lichenysin with C$_3$(CA)$_2$ and CAM, as well as the effect of these mixtures against a monolayer of a total lipid extract from
E. coli (TLE), was studied. The TLE, rich in phosphatidylethanolamine and phosphatidylglycerol, was used as a simplified model of an *Enterobacteriaceae* membrane.

The $\pi$-A isotherms of mixed monolayers at 37°C with C$_3$(CA)$_2$ are shown in Figure 2A. The C$_3$(CA)$_2$ isotherm is not shown because the gemini surfactant is partially soluble and on compression dissolves in the subphase. The lichenysin isotherm shows a profile with a starting gaseous phase and an expanded liquid phase until monolayer collapse. On the other hand, the TLE isotherm has a short expanded liquid phase followed by a condensate liquid phase until collapse. The isotherm of the C$_3$(CA)$_2$:lichenysin binary mixture shows a liquid condensate and collapsed phases. The fact that the surface pressure ($\pi$) at collapse of C$_3$(CA)$_2$:lichenysin is higher than that of either component indicates a higher number molecules on the monolayer, which might be interacting to form a mixed or catanionic aggregate. Isotherms of binary systems of TLE with C$_3$(CA)$_2$ and TLE with lichenysin (Figure 2A) are almost parallel and have a lower $\pi$ at collapse than the isotherm of TLE alone. This indicates that there are fewer molecules at the interface because of the formation of mixed aggregates that solubilize into the subphase. The isotherms of TLE and TLE:lichenysin are also included in Figure 2B for clarity. Comparing the mixed isotherm of the three components with the isotherm of TLE, it can be seen that they have the same profile, with a slight increase of the $\pi$ at collapse. Given that the $\pi$ of the TLE:C$_3$(CA)$_2$:lichenysin isotherm at collapse is also higher than those of the monolayers corresponding to the studied binary systems, TLE:lichenysin and TLE:C$_3$(CA)$_2$, we propose that the mixture C$_3$(CA)$_2$:lichenysin has a synergic effect, albeit slight, when mixed with TLE, which indicates that C$_3$(CA)$_2$ forms catanionic aggregates with lichenysin.
Figure 2. Isotherm curves, surface pressure – mean molecular area (π-A), of: (triangles) total lipid extract of *E. coli* (TLE), (empty circles) TLE:Lichenysin, (empty squares) TLE:Surfactant, (crosses) TLE:Surfactant:Lichenysin, (filled squares) Lichenysin 20%, (filled circles) Surfactant:Lichenysin 20%. Surfactants are C$_3$(CA)$_2$ (A) and CAM (B).

To compare the gemini surfactant mixtures with those of a single-chain surfactant, the same experiments were performed with CAM, the monomeric counterpart of C$_3$(CA)$_2$. The π-A isotherms of mixed monolayers at 37ºC with CAM are shown in Figure 2B. Like C$_3$(CA)$_2$, CAM is also soluble in water and its isotherm is not shown. The binary CAM:lichenysin isotherm shows a short expanded liquid phase and a condensate liquid phase with a high π at collapse. Similarly to C$_3$(CA)$_2$, CAM forms catanionic aggregates with lichenysin. However, contrary to the C$_3$(CA)$_2$ results, the isotherm curve of the monolayer of the three components (TLE:CAM:lichenysin) shows a π at collapse lower than TLE alone and lower than the isotherm of CAM:lichenysin. This indicates a lower number of molecules in the monolayer or that the molecules remaining in the monolayer have less surface activity.
As explained before, one molecule of the cationic surfactants $C_3(CA)_2$ or CAM interacts with one molecule of the anionic lichenysin. The resulting catanionic aggregates would be cationic and non-ionic, respectively, and in the case of $C_3(CA)_2$, interactions with the partially negatively-charged phospholipids of TLE would be preferred. The formation of a catanionic aggregate with a different charge may explain the differences in the monolayer behaviour.

A complementary analysis of the data obtained was performed in order to understand the interactions and miscibility of the components by calculating the excess of free energy of the mixtures, $\Delta G_m^{\text{ex}}$. If all the components mix ideally, at a given surface pressure and temperature, the proportional sum of areas of single component isotherms would be equal to the experimental area of the mixed monolayer isotherm. Any deviation would be due to interactions between components and partial or total miscibility. Hence, in a binary system, an ideal behaviour without miscibility of any component would result in $\Delta G_m^{\text{ex}}=0$, while any deviation from it will be due to molecular interactions, even with the subphase, or the miscibility of each component or mixed aggregates. A negative value of the excess of free energy evidences strong interactions of the two components, which leads to a partial formation of miscible complexes or aggregations that are diluted in the subphase, whose quantity depends on the molar ratio of the components. On the other hand, a positive value of the excess free energy evidences that the interactions between the two components are weaker than the individual self-interactions. In this case, at least one component forms auto-aggregates and becomes diluted in the subphase. $\Delta G_m^{\text{ex}}$ values for $C_3(CA)_2$, CAM and their mixtures with lichenysin and TLE are shown in Figure 3.
Figure 3. Excess of free energy, $\Delta G_{m}^{\text{ex}}$ (J/mol), of mixtures based in $\text{C}_3(\text{CA})_2$ (A) and CAM (B).

Mixtures: Total lipid extract(TLE):Surfactant:Lichenysin (red), TLE:Lichenysin (yellow), TLE:Surfactant (green) and Surfactant:Lichenysin (blue).

The binary mixtures with $\text{C}_3(\text{CA})_2$ ($\text{C}_3(\text{CA})_2$:lichenysin and TLE:$\text{C}_3(\text{CA})_2$) both have positive $\Delta G_{m}^{\text{ex}}$ values, of 6016.06 and 1337.48 J/mol, respectively (Figure 3A), indicating that $\text{C}_3(\text{CA})_2$ forms auto-aggregates and solubilizes in the subphase. Although auto-aggregation of $\text{C}_3(\text{CA})_2$ is thermodynamically more favoured, the $\pi$-$\Lambda$ isotherm of $\text{C}_3(\text{CA})_2$:lichenysin (Figure 2A) shows that part of $\text{C}_3(\text{CA})_2$ forms stable catanionic aggregates with lichenysin that remain in the monolayer. Given that one molecule of lichenysin partially neutralizes a molecule of $\text{C}_3(\text{CA})_2$ and the molar ratio for this binary mixture is 8:2, there would be free molecules of $\text{C}_3(\text{CA})_2$. The auto-aggregation of the excess of $\text{C}_3(\text{CA})_2$ in the mixture might explain the higher $\Delta G_{m}^{\text{ex}}$ value. The $\Delta G_{m}^{\text{ex}}$ of the binary mixture TLE:lichenysin is -2494.57 J/mol. The negative value indicates the formation of mixed aggregates that partially solubilize in the subphase. The $\Delta G_{m}^{\text{ex}}$ of the three-component mixture is 1451.49 J/mol, indicating that the solubilization of the resulting catanionic mixed aggregates in the subphase is not relevant, thus the catanionic aggregate $\text{C}_3(\text{CA})_2$:lichenysin is stable and remains in the phospholipid monolayer.

Values of $\Delta G_{m}^{\text{ex}}$ for CAM and its mixtures are shown in Figure 3B. The mixture with lichenysin has a $\Delta G_{m}^{\text{ex}}$ (4460.99 J/mol) similar to that of $\text{C}_3(\text{CA})_2$:lichenysin, and the positive value can be
attributed to the solubilization of the excess of CAM. The mixture with TLE has a $\Delta G_m^{ex}$ value near to zero, 258.39 J/mol, which suggests that although a small part of CAM is solubilized, its mixture forms a very stable monolayer. Finally, the $\Delta G_m^{ex}$ of the three-component mixture with CAM is negative, -771.53 J/mol, unlike the three-component mixture with $C_3(CA)_2$, which indicates that all or some of its components form mixed aggregates that solubilize in the subphase and are not present in the monolayer. In $\pi$-$A$ isotherms (Figure 2B), this reduction of molecules in the monolayer corresponds to the reduction of surface pressure.

Our hypothesis is that the mixture of TLE, $C_3(CA)_2$ and lichenysin has a synergic effect, since the gemini surfactant is able to remain in the phospholipid monolayer when partially neutralized by lichenysin. Also, the comparison with CAM suggests it might be caused by the remaining positive charge of the gemini surfactant, because CAM, completely neutralized by lichenysin, is stable when mixed with TLE but not when mixed with both TLE and lichenysin. In conclusion, the free cationic charge of the catanionic aggregate $C_3(CA)_2$:lichenysin allows it to become attached to the phospholipids of the TLE.

Notably, the catanionic mixtures studied in this work can be considered green systems. For practical reasons, lichenysin was synthesized using commercial glucose as the carbon source, but this can be substituted by molasses. The gemini surfactant $C_3(CA)_2$ was synthesized using renewable raw materials, arginine and fatty acid, by a chemoenzymatic approach in which papain is deposited into cells. The formulations were prepared without high mechanical energy, and moreover, biodegradation studies showed that CAM and $C_3(CA)_2$ are readily biodegradable surfactants.

**Minimal inhibitory concentration (MIC).** The antimicrobial activity of lichenysin, and its catanionic mixtures with CAM and $C_3(CA)_2$ was determined (Table 1). As previously reported by
Coronel-León et al., lichenysin was not active against the microorganisms tested (MIC≥1000μM). This might be due to the null capacity of lichenysin to disrupt the cell envelope, which acts as a selective barrier for a wide range of solutes, perhaps because lichenysin is an anionic molecule, like the cell envelope itself. A successful attachment is thought to usually require a proper electrostatic interaction between the cell envelope and the antimicrobial molecule: lipopolysaccharide in the case of Gram-negative bacteria or lipoteichoc acid in Gram-positive bacteria. This interaction would be possible with arginine-based surfactants, which are cationic amphiphiles, and accordingly C₃(CA)₂ and CAM showed antimicrobial activity (Table 1). C₃(CA)₂ was the most effective against both Gram-negative and Gram-positive bacteria and the yeast C. albicans. Gram-negative bacteria are usually only inhibited by relatively high concentrations of a cationic surfactant, since the presence of lipopolysaccharide in the outer membrane makes it more difficult for amphiphiles to diffuse across. Its monomeric counterpart, CAM, showed only a moderate antimicrobial activity against Gram-positive bacteria (Table 1).

The MIC of the catanionic mixtures was characterized in the search for antimicrobial synergies. Based on previous work, a molar ratio of 8:2 was chosen. The fractional inhibitory concentration (FIC) was used to detect possible synergies in the catanionic mixtures. The only mixture that showed a synergy was C₃(CA)₂:lichenysin 8:2 (mol:mol). MICs against Y. enterocolitica, B. subtilis, E. coli O157:H7 and C. albicans showed FIC values of 0.1, 0.2, 0.4 and 0.4, respectively (Table 1). On the other hand, the mixture CAM:lichenysin 8:2 (mol:mol) did not show any synergy in its antimicrobial activity. The difference between the gemini and single-chain surfactants is the presence of two positive charges instead of one. As demonstrated, one charge of each molecule is neutralised by the negative charge of lichenysin and consequently, the catanionic aggregate
presents a synergic antimicrobial activity only if it has a cationic character after the neutralization by lichenysin.

It can also be observed that formulations with the gemini surfactant were active against methicillin-resistant S. aureus, although a synergy was not characterized (Table 1). One of the major challenges to healthcare in the 21st century is the emergence of multi drug-resistant bacteria. Strategies to minimize this danger include an extensive education about the risks of inappropriate antibiotic use. Additionally, a few new antibiotics are currently in clinical development.

Due to the promising antimicrobial behaviour of the C₃(CA)₂:lichenysin catanionic mixture, it was chosen for further studies with the microorganisms against which it had exhibited antimicrobial synergies: Y. enterocolitica, B. subtilis, E. coli O157:H7 and C. albicans.

| Microorganisms         | MIC  | MIC  | MIC  | FIC  | MIC  | FIC  | TI   | TI   | TI   |
|------------------------|------|------|------|------|------|------|------|------|------|
|                        | Lich| C₃(CA)₂| Mixture| CAM| Lich| CAM| Mixture| Lich| C₃(CA)₂| Mixture|
| E. coli ATCC 25922     | 1000 | 15.7 | 15.7 | 0.8 | 1000 | >500 | 1.0  | 0.01 | 0.85 | 1.41 |
| E. coli O157:H7 CECT 4267 | >1000 | 31.3 | 15.7 | 0.4 | 1000 | >500 | 1.0  | <0.01 | 0.43 | 1.41 |
| P. aeruginosa ATCC 27853 | >1000 | 31.2 | 31.2 | 0.8 | 1000 | >500 | 1.0  | <0.01 | 0.43 | 0.71 |
| K. pneumoniae ATCC 13883 | >1000 | >250 | >250 | 0.9 | 1000 | >500 | 1.0  | <0.01 | <0.05 | <0.09 |
| Y. enterocolitica ATCC 9610 | >1000 | 62.5 | 7.8  | 0.1 | 1000 | >500 | 1.8  | <0.01 | 0.21 | 2.84 |
| S. aureus ATCC 29213   | 1000 | 7.8  | 7.8  | 0.8 | 250  | 500  | 3.4  | 0.01 | 1.71 | 2.84 |
| MRSA ATCC 43300        | 1000 | 3.9  | 15.7 | 3.2 | 250  | 500  | 1.6  | 0.01 | 3.41 | 1.41 |
| S. epidermis ATCC 12228 | 1000 | 3.9  | 3.9  | 0.8 | 125  | 125  | 6.6  | 0.01 | 3.41 | 5.68 |
| K. rhizophila ATCC 9341 | >1000 | 3.9  | 3.9  | 0.8 | 250  | 500  | 1.8  | <0.01 | 3.41 | 5.68 |
| B. subtilis ATCC 6633   | >1000 | 15.7 | 3.9  | 0.2 | 250  | 250  | 1.8  | <0.01 | 0.85 | 5.68 |
Table 1. MIC values (μM) of lichenysin, C₃(CA)₂, the mixture C₃(CA)₂:lichenysin 8:2 (mol:mol), CAM and the mixture CAM:lichenysin 8:2 (mol:mol); FIC values of the mixtures; and therapeutic indices (TI) of lichenysin, C₃(CA)₂ and the mixture C₃(CA)₂:lichenysin 8:2 (mol:mol).

|                      | L. monocytogenes ATCC 15313 | C. albicans ATCC 10231 |
|----------------------|-----------------------------|------------------------|
|                      | 1000                        | >1000                   |
|                      | 3.9                         | 15.7                   |
|                      | 3.9                         | 7.8                    |
|                      | 0.8                         | 0.4                    |
|                      | 500                         | 500                    |
|                      | >500                        | >500                   |
|                      | 0.8                         | 1.0                    |
|                      | 0.01                        | <0.01                  |
|                      | 3.41                        | 0.85                   |
|                      | 5.68                        | 2.84                   |

Viability study by cell count and flow cytometry. To establish the time required by C₃(CA)₂:lichenysin 8:2 (mol:mol) to show significant antimicrobial activity against each microorganism, a viability assay was performed by incubating Y. enterocolitica, E. coli O157:H7, B. subtilis, and C. albicans in BPW containing the catanionic mixture at MIC values. The time when their viability in solid media was reduced by 90% was established as a point of reference for the FC and electron microscopy assays. Incubations of Y. enterocolitica, B. subtilis and C. albicans reached this point at 150, 105 and 90 minutes, respectively. The cell count from E. coli O157:H7 incubation remained stable for 150 minutes, which might evidence a bacteriostatic effect. An arbitrary time of 90 minutes was chosen for the FC assay.

The aim of the FC assay, using fluorescent staining reagents PI and BOX, was to determine how the surfactant antimicrobial activity affected the microbial cells. BOX-stained cells are associated with a depolarization of the cytoplasmic membrane, which is a reversible process, while PI-stained cells indicate pore formation and permeation of the cytoplasmic membrane, which causes cell death.

No strain showed cells stained only with PI after treatments with C₃(CA)₂ or its mixture with lichenysin (Figure 4). In contrast, cells stained only with BOX, as well as with both BOX and PI, were identified. The absence of only PI-stained cells might be explained because the action of both
C₃(CA)₂ and its mixture with lichenysin against cytoplasmic membranes involved a depolarization before permeation. This hypothesis matches the already observed unspecific mode of action of cationic surfactants in the disruption of the Gram-negative bacterial cell envelope: firstly disrupting the integrity of the outer membrane by the electrostatic interaction between the cationic surfactant and the anionic cell envelope, and then disintegrating the cell inner membrane through the hydrophobic interaction between the hydrocarbon chains of the surfactants and the bacterial membrane phospholipids. The subsequent cytoplasm leakage eventually leads to the death of bacteria.²⁶

![Figure 4](image)

**Figure 4.** Reduction of viability (Viab) and flow cytometry (FC) results of treatments with C₃(CA)₂:Lichenysin 8:2 (mol:mol) (mixture) and C₃(CA)₂ at the corresponding MIC and without treatment (control), against (A) *Y. enterocolitica* (150 min), (B) *E. coli* O157:H7 (90 min), (C) *B. subtilis* (105 min) and (D) *C. albicans* (90 min). Viability assay: nonculturable (red) and culturable (blue). Flow cytometry: BOX- and PI-stained (orange), BOX-stained (yellow), and non-stained
(white). Areas between dotted black lines correspond to the proportion of viable but nonculturable cells.

*Y. enterocolitica* (Figure 4A) showed a similar reduction of viability in solid media after being in contact with the catanionic mixture or C₃(CA)₂ (77.9±0.4 and 78.1±8.7%, respectively). In contrast, according to the FC results, the proportion of cells with a permeated and depolarized cell envelope was only 20.8±2.5% after treatment with the catanionic mixture, and 59.1±4.8% for the gemini surfactant. Interestingly, the amount of permeated cells (stained with both reagents) was similar after treatment with the mixture (17.2±2.5%) or C₃(CA)₂ (13.9±1.3%), which indicates the mixture is more efficient than the single surfactant, given that the MIC is reduced from 62.5 to 7.8 μM.

Although a high proportion of *Y. enterocolitica* cells were not stained, suggesting that their cell envelope was unaltered, they were not culturable in solid media. An explanation might be that other alterations, not detected by FC, rendered the cells unable to recover in solid media. Such cells are considered to be in a transitory viable but nonculturable (VBNC) state and represented 57.2% of the cells treated with the mixture and 19% of those treated with C₃(CA)₂. The VBNC state is associated with sub-lethal metabolic and genetic alterations in response to a stressful environment. The higher percentage of VBNC cells after treatment with the mixture indicates that this treatment affected the cell envelope less than the gemini surfactant, but reduced the viability alike.

Results of *E. coli* O157:H7 (Figure 4B) differed from those of *Y. enterocolitica* despite both being *Enterobacteriaceae*. Almost all the cells grew on solid media, while only 13.4±19% of cells treated with C₃(CA)₂ were nonculturable. In the FC assay, 11.5±0.4 and 9±1% of cells were stained when incubated with the catanionic mixture or C₃(CA)₂, respectively, indicating an altered
cytoplasmic membrane. Both the reduction of viability and the FC results closely match those of the corresponding negative control. This strengthens the hypothesis that $C_3(CA)_2$ has a bacteriostatic effect against $E. coli$ O157:H7, which is not significantly improved when mixed with lichenysin.

Viability results for $B. subtilis$ (Figure 4C) show a similar reduction after treatments with the catanionic mixture and $C_3(CA)_2$ (near 100%). According to the FC results, the proportion of cells with a permeated or depolarized membrane was low, as in $Y. enterocolitica$. A significant difference between the proportion of cells affected by the mixture (38.4±3.5%) and $C_3(CA)_2$ (59.94±7.3%) at their corresponding MIC was also observed. The lower proportion of non-stained cells with respect to the nonculturable cells again indicates the presence of VBNC cells: 57.2% when treated with the mixture and 40.1% when treated with $C_3(CA)_2$.

Finally, all treatments exerted a strong effect against $C. albicans$ (Figure 4D) at 90 minutes, with a near 100% reduction of viability. The FC results were similar in both treatments: almost 100% of cells had a permeated cell envelope when treated with the catanionic mixture or the gemini surfactant. Thus, an interesting antifungal activity of $C_3(CA)_2$ was observed, but with such high values it is impossible to know if lichenysin improved the mortality effect.

**Transmission electronic microscopy (TEM).** Ultrastructural alterations caused by $C_3(CA)_2$:lichenysin 8:2 (mol:mol) and $C_3(CA)_2$ in the most affected microorganisms, $Y. enterocolitica$, $B. subtilis$ and $C. albicans$, were identified by TEM.
Figure 5. Transmission electronic microscopy images from (A) *Y. enterocolitica* (x50000), (B) *B. subtilis* (x40000) and (C) *C. albicans* (x20000) after 90 minutes contact with (1) C₃(C₅A)₂:lichenysin (7.8, 3.9 and 7.8 μM respectively) and (2) C₃(C₅A)₂ (62.5, 15.7 and 15.7 μM respectively).

As shown in Figure 5A.1, most *Y. enterocolitica* cells incubated with the surfactant mixture had an intact cell envelope. Nevertheless, some cells were secreting outer membrane vesicles, and chromatin condensation was observed, although this has been reported as a possible artefact of the technique. When cells were treated with C₃(C₅A)₂ at its MIC (Figure 5A.2), a high amount of cellular waste was observed, due to cell lysis. The majority of cells showed cytoplasm condensation near the cytoplasmic membrane. In addition, the cell wall was clearly thinner than in the control (not shown). All these alterations might explain the reduction of viability (Figure 4A) of cells treated with C₃(C₅A)₂ at its MIC. Overall, the most significant alteration of mixture-treated *Y. enterocolitica* cells was the formation of outer membrane vesicles, which has been...
recently associated with a Gram-negative bacteria stress response.\textsuperscript{30,31} These may be initial alterations eventually leading to the cell envelope disruption observed in $C_3(CA)_2$-treated cells, which received a much higher concentration of the gemini surfactant. In the FC results for \textit{Y. enterocolitica} cells (Figure 4A), the large difference in the proportion of BOX-stained cells between treatments (3.5\% for the mixture versus 45.3\% for $C_3(CA)_2$) could be explained by the reduction in the cell wall width apparent in the $C_3(CA)_2$-treatment images (Figure 5A.2), with a consequent greater membrane depolarization in cells not yet lysated. Although the mixture caused less severe alterations than $C_3(CA)_2$, it is clear that both had the same impact on cell viability. $C_3(CA)_2$ reduced the viability by disrupting the cell envelope, while lichenysin might be acting at a cytoplasmic level, which would keep the cell from growing or recovering, and would explain the larger proportion of VBN$^C$ cells after the mixture treatment (Figure 4A).

The effect visible in \textit{B. subtilis} (Figure 5B) was milder than in \textit{Y. enterocolitica} cells. Cells treated with the mixture or with $C_3(CA)_2$ alone showed a slight dilution of the cytoplasm. In both cases, empty cells were detected, but they were also observed in control images. Although the cytoplasm seems slightly altered, the cell wall was not broken or thinned. This could be related to the low proportion of cells stained only by BOX in both treatments, indicating few depolarized cells (Figure 4C).

Finally, \textit{C. albicans} (Figure 5C) gave interesting results. First of all, no alteration in the cell wall was visible, neither with the mixture nor with $C_3(CA)_2$, but the cytoplasm was severely affected. None of the cells presented nuclei, perhaps because they had been disrupted, or vacuoles, which were always present in control cells (not shown). And finally, numerous cells had begun the formation of one or more gems, all of which seem to have been interrupted at the same point, while in the control the gemmating processes had been stopped at different stages by the fixation
procedure. Overall, the severe alterations of the cytoplasm seem to have caused the cell death detected in both the viability reduction and FC assays (Figure 4D).

The microbial cell envelope acts as an effective permeability barrier against antibiotics or biocides. Some compounds with little or no antimicrobial activity are being used to block or bypass active or intrinsic bacterial resistance mechanisms or enhance antibiotic action to rescue the activity of existing drugs. These compounds are called antibiotic adjuvants. A cationic molecule able to interact with a negatively charged bacterial cell envelope causing destabilization and permeability could allow antimicrobial compounds to enter the cell. According to the results obtained, the catanionic mixture could fulfill this role and may be considered a possible antibiotic adjuvant to reduce the onset of resistance.

**RBC hemolysis and therapeutic index.** In order to test the irritancy potential of lichenysin, C₃(CA)₂ and their 8:2 mixture (mol:mol), the hemolysis level of these surfactants was studied. The resulting hemolysis curves are shown in Figure 6 and values extracted from them in Table 2.

|             | H₅₀ (µM) | DI (%) | IP        |
|-------------|----------|--------|-----------|
| Lichenysin  | 8.0      | 22.6   | 0.37 (Irritant) |
| C₃(CA)₂     | 13.3     | 36.9   | 0.28 (Irritant) |
| Mixture     | 22.1     | 25.8   | 0.70 (Irritant) |

**Table 2.** Hemolysis (H₅₀), denaturation index (DI) and irritant potential (IP) of lichenysin, C₃(CA)₂ and their mixture at a molar ratio 8:2 (mol:mol) after 20 minutes.

All the studied surfactant formulations were classified as irritants (Table 2). Among them, the mixture was the least irritant and had the highest H₅₀ (22.1 µM), which was higher than its
estimated CMC (18 μM), making it the least hemolytic. Despite the low DI of lichenysin (22.6%), which should favour a less severe irritancy, it was also characterised as irritant. This can be explained by the low H50 of lichenysin (8.0 μM), resulting in a high hemolytic activity below its CMC (14.4 μM). Coronel-León et al. demonstrated that the presence of cholesterol in the lipid membrane, typical of eukaryotic cells, enhances lichenysin-induced leakage of cytoplasm.24 Finally, C₃(CA)₂ had a high hemolytic activity at very low concentrations (13.3 μM), considering that its CMC is 4.3 mM.

Having determined the superior antimicrobial activity of the catanionic mixture, the relationship between the hemolysis curves and their respective MICs against different microorganisms was key for establishing whether this heightened activity affects eukaryotic cells. Lichenysin showed no antimicrobial activity within the interval shown, so no MIC was drawn (Figure 6A). The MICs of C₃(CA)₂ (Figure 6B) and the mixture (Figure 6C) against Gram-positive bacteria were lower than against Gram-negative bacteria. When MIC values are compared with the hemolysis results, two observations stand out. First, the MICs of C₃(CA)₂ against Gram-negative bacteria are over the H50, which is the case in only a few of the MICs of the mixture. Secondly, all the MICs of the mixture against Gram-positive bacteria and C. albicans are below 20% of hemolysis, which is not achieved by the gemini surfactant.
The therapeutic index (TI) correlates MICs with the $H_{50}$ to express the relative cell selectivity of the formulations against microorganisms (Table 1). TI values of lichenysin were the lowest, due to its lack of antimicrobial activity. Overall, TI values of the catanionic mixture were higher than those of $C_3(CA)_2$ for two possible reasons: the MICs of the mixture were lower, implying a higher antimicrobial activity, or the $H_{50}$ was higher, resulting in less hemolytic activity. In this case, it has already been proven that the mixture is less hemolytic, which would improve the TI when MICs are equal. However, in addition, a synergic antimicrobial activity against determined strains was detected. Focusing on *E. coli* O157:H7, *Y. enterocolitica*, *B. subtilis* and *C. albicans*, it can be seen that the TIs of the mixture formulation improved at least three-fold compared to $C_3(CA)_2$. It was therefore more selective against microbial cells than against eukaryotic cells, while the other formulations were less selective.

**CONCLUSIONS**

The green catanionic mixture of $C_3(CA)_2$, a cationic arginine-based gemini surfactant, and lichenysin, an anionic cyclic lipopeptide biosurfactant, induces the formation of a catanionic aggregate with a significant synergic antimicrobial activity, in which lichenysin acts as an antimicrobial potentiator.

The partial neutralisation of the two cationic polar heads of the gemini surfactant by the anionic charge of the biosurfactant changes its mode of action. When cells are treated with the gemini surfactant alone, the cell envelope is first depolarized and finally disrupted. When cells are treated
with the catanionic mixture, bacterial cell envelopes are not disrupted but cells are altered at a cytoplasmic level, which makes them more likely to enter a viable but nonculturable state. Additionally, the catanionic mixture showed a strong fungicidal activity. Our hypothesis is that the free cationic charge allows the catanionic aggregate to approach the anionic cell envelope by electrostatic interactions and to interact with it by aggregation with the bacterial phospholipids, which enhances the antimicrobial effect. On the other hand, when the cationic surfactant is completely neutralised, as occurs in catanionic mixtures of lichenysin and monomeric arginine-based surfactants, no synergic antimicrobial activity is detected. Finally, the therapeutic index of the catanionic mixture, and thus its selectivity, is better than that of the gemini surfactant because it has a reduced hemolytic activity at the minimal inhibitory concentration, although it is still considered irritant. This study offers new insights into the potential advantages of environmentally friendly catanionic mixtures of green surfactants with improved surface-antimicrobial properties for biotechnological applications.

AUTHOR INFORMATION

Corresponding Author

*E-mail: ammarques@ub.edu

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

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REFERENCES

(1) Marchant, R.; Banat, I.M. Microbial Biosurfactants: Challenges and Opportunities for Future Exploitation. Trends Biotechnol. 2012, 30, 558-565.

(2) Lundberg, D.; Unga, J.; Galloway, A.L.; Menger, F.M. Studies on an Ester-modified Cationic Amphiphile in Aqueous Systems: Behavior of Binary Solutions and Ternary Mixtures with Conventional Surfactants. Langmuir 2007, 23, 11434-11442.

(3) Brycki, B.; Szulc, A. Gemini alkyldeoxy-D-glucitolammonium Salts as Modern Surfactants and Microbicides: synthesis, antimicrobial and surface activity, biodegradation. PLoS One 2014, 9, e84936.

(4) Kamech, N.; Vukicevic, D.; Ladram, A.; Piesse, C.; Vasseur, J; Bojovic, V.; Simunic, J.; Juretic, D. Improving the Selectivity of Antimicrobial Peptides from Anuran Skin. J. Chem. Inf. Model. 2012, 52, 3341-3351.

(5) Pinazo, A.; Manresa, M.A.; Marqués, A.M.; Bustelo, M.; Espuny, M.J.; Pérez, L. Amino Acid-based Surfactants: New Antimicrobial Agents. Adv. Colloid Interface Sci. 2016, 228, 17-39.

(6) Shekhar, S.; Sundaramanickam, A.; Balasubramanian, T. Biosurfactant Producing Microbes and their Potential Applications: a Review. Crit. Rev. Environ. Sci. Technol. 2015, 45, 1522-1554.
(7) Li, Y.; Yang, S.; Mu, B. The Surfactin and Lichenysin Isoforms Produced by *Bacillus licheniformis* HSN 221. *Anal. Lett.* **2010**, *43*, 929-940.

(8) Coronel-León, J.; de Grau, G.; Grau-Campistany, A.; Farfan, M.; Rabanal, F.; Manresa, A.; Marqués, A.M. Biosurfactant Production by AL1.1, a *Bacillus licheniformis* Strain Isolated from Antarctica: Production, Chemical Characterization and Properties. *Ann. Microbiol.* **2015**, *65*, 2065-2075.

(9) Llarch, À.; Logan, N.A.; Castellví, J.; Prieto, M.J.; Guinea, J. Isolation and Characterization of Thermophilic *Bacillus* spp. from Geothermal Environments on Deception Island, South Shetland Archipelago. *Microb. Ecol.* **1997**, *34*, 58-65.

(10) Tondre, C.; Caillé, C. Properties of the Amphiphilic Films in Mixed Cationic/Anionic Vesicles: a Comprehensive View from a Literature Analysis. *Adv. Colloid Interface Sci.* **2001**, *93*, 115-134.

(11) Pucci, C.; Pérez, L.; La Mesa, C.; Pons, R. Characterization and Stability of Catanionic Vesicles Formed by Pseudo-tetraalkyl Surfactant Mixtures. *Soft Matter* **2014**, *10*, 9657-9667.

(12) Coronel-León, J.; Pinazo, A.; Pérez, L.; Espuny, M.J.; Marqués, A.M.; Manresa, A. Lichenysin-Geminal Acid-based Surfactants: Synergistic Action of an Unconventional Antimicrobial Mixture. *Colloids Surf., B* **2017**, *149*, 38-47.

(13) Infante M.R.; García-Dominguez J.J.; Erra P.; Julià M.R.; Prats M. Surface Active Molecules: Preparation and Properties of Long Chain n⁴-acyl-l-α-amino-ω-guanidine Alkyl Acid Derivatives. *Int. J. Cosmet. Sci.* **1984**, *6*, 275-282.
(14) Pérez, L.; Torres, J.L.; Manresa, A.; Solans, C.; Infante, M.R. Synthesis, Aggregation, and Biological Properties of a New Class of Gemini Cationic Amphiphilic Compounds from Arginine, bis(Args). *Langmuir* **1996**, *12*, 5296-5301.

(15) Lozano, N.; Pérez, L.; Pons, R.; Luque-Ortega, J.R.; Férnandez-Reyes, M.; Rivas, L.; Pinazo, A. Interaction Studies of Diacyl Glycerol Arginine-based Surfactants with DPPC and DMPC Monolayers, Relation with Antimicrobial Activity. *Colloids Surf., A* **2008**, *319*, 196-203.

(16) Gaines Jr., G.L. Thermodynamic Relationships for Mixed Insoluble Monolayers. *J. Colloid Interface Sci.* **1966**, *21*, 315-319.

(17) Saiman, L. Clinical Utility of Synergy Testing for Multidrug-resistant *Pseudomonas aeruginosa* Isolated From Patients with Cystic Fibrosis: “the motion for”. *Paediatric respiratory reviews* **2007**, *8*, 249-255.

(18) Colomer, A.; Perez, L.; Pons, R.; Infante, M.R.; Perez-Clos, D.; Manresa, A.; Espuny, M.J.; Pinazo, A. Mixed Monolayer of DPPC and Lysine-based Cationic Surfactants: an Investigation into the Antimicrobial Activity. *Langmuir* **2013**, *29*, 7912-7921.

(19) Pape, W.J.W.; Hoppe, U. Standardization of an In Vitro Red Blood Cell Test for Evaluating the Acute Cytotoxic Potential of Tensides. *Arzneimittelforschung/Drug resistances* **1990**, *40*, 498-502.
(20) Maget-Dana, R. The Monolayer Technique: a Potent Tool for Studying the Interfacial Properties of Antimicrobial and Membrane-lytic Peptides and their Interactions with Lipid Membranes. *Biochim. Biophys. Acta* **1999**, 1462, 109-140.

(21) Coronel-León, J.; Marqués, A.M.; Bastida, J.; Manresa, A. Optimizing the Production of the Biosurfactant Lichenysin and its Application in Biofilm Control. *J. Appl. Microbiol.* **2015**, 120, 99-111.

(22) Piera, E.; Infante, M.R.; Clapés, P. Chemo-enzymatic Synthesis of Arginine-based Gemini Surfactants. *Biotechnol. Bioeng.* **2000**, 70, 323-331.

(23) Pérez, L.; Pinazo, A.; Vinardell, P.; Clapés, P.; Angelet, M.; Infante M.R. Synthesis and Biological Properties of Dicationic Arginine-diglycerides. *New J. Chem.* **2002**, 26, 1221-1227.

(24) Coronel-León, J.; Aranda, F.J.; Teruel, J.A.; Marqués, A.; Manresa, A.; Ortiz, A. Kinetic and Structural Aspects of the Permeabilization of Biological and Model Membranes by Lichenysin. *Langmuir* **2016**, 32, 78-87.

(25) Uppu, D.S.S.M.; Haldar, J. Lipopolysaccharide Neutralization by Cationic-Amphiphilic Polymers through Pseudoaggregate Formation. *Biomacromolecules* **2016**, 17, 862-873.

(26) Zhou, C.; Wang, F.; Chen, H.; Li, M; Qiao, F.; Liu, Z.; Hou, Y.; Wu, C.; Fan, Y.; Liu, L.; Wang, S.; Wang, Y. Selective Antimicrobial Activities and Action Mechanism of Micelles Self-assembled by Cationic Oligomeric Surfactants. *ACS Appl. Mater. Interfaces* **2016**, 8, 4242-4249.
(27) Bouhdid, S.; Abrini, J.; Amensour, M.; Zhiri, A.; Espuny, M.J.; Manresa A. Functional and Ultrastructural Changes in *Pseudomonas aeruginosa* and *Staphylococcus aureus* Cells Induced by *Cinnamomum verum* Essential Oil. *J. Appl. Microbiol.* **2010**, 109, 1139-1149.

(28) Pinto, D.; Santos, M.A.; Chambel, L. Thirty Years of Viable but Nonculturable State Research: Unsolved Molecular Mechanisms. *Crit. Rev. Microbiol.* **2015**, 41, 61-76.

(29) Nevot, M.; Deroncele, V.; López-Iglesias, C.; Bozal, N.; Guinea, J.; Mercade, E. Ultrastructural Analysis of the Extracellular Matter Secreted by the Psychrotolerant Bacterium *Pseudoalteromonas antarctica* NF3. *Microb. Ecol.* **2006**, 51, 501-507.

(30) McBroom, A.J.; Kuehn, M.J. Release of Outer Membrane Vesicles by Gram-negative Bacteria is a Novel Envelope Stress Response. *Mol. Microbiol.* **2007**, 63, 2, 545-558.

(31) Schwechheimer, C.; Kulp, A.; Kuehn, M.J. Modulation of Bacterial Outer Membrane Vesicle Production by Envelope Structure and Content. *BMC Microbiol.* **2014**, 14, 324.

(32) Wright, G.D. Antibiotic Adjuvants: Rescuing Antibiotics from Resistance. *Trends Microbiol.* **2016**, 24, 862-871.

(33) Zabawa, T.P.; Pucci, M.J.; Parr Jr., T.R.; Lister, T. Treatment of Gram-negative Bacterial Infections by Potentiation of Antibiotics. *Curr. Opin. Microbiol.* **2016**, 33, 7-12.
