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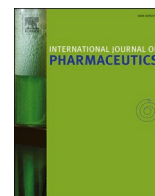
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(E, E)-farnesol and myristic acid-loaded lipid nanoparticles overcome colistin resistance in *Acinetobacter baumannii*

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ABSTRACT

The rise of colistin-resistant *Acinetobacter baumannii* has severely limited treatment options for infections caused by this pathogen. While terpene alcohols and fatty acids have shown potential to enhance colistin's efficacy, but their high lipophilicity limits their clinical application. To address this, we developed water-dispersible lipid nanoparticles (LNPs) in two sizes (40 nm and 130 nm), loaded with these compounds to act as colistin adjuvants. Among eleven LNP formulations, six significantly reduced colistin's minimum inhibitory concentration (MIC) by 16- to 64-fold. The most effective, featuring (E,E)-farnesol and myristic acid, were further examined for bactericidal activity, membrane disruption, cytotoxicity, and *in vivo* efficacy in *Galleria mellonella* larvae. Time-kill studies demonstrated that at an adjuvant concentration of 60 mg/L, these LNPs eradicated bacteria when combined with 4 mg/L free colistin for resistant isolates (MIC = 128 mg/L) and 0.06 mg/L for susceptible isolates (MIC = 0.5 mg/L), without regrowth. Myristic acid-loaded LNPs combined with free colistin at 1/8 MIC resulted in a 4.2-fold higher mortality rate than the combination with (E,E)-farnesol-loaded LNPs in resistant strains. This result was correlated with a 45-fold faster increase in inner membrane permeability, measured by propidium iodide (PI) uptake, in the presence of myristic acid-loaded LNPs compared with a 13-fold faster increase with (E, E)-farnesol-loaded LNPs. DiSC3(5) assays revealed that LNPs alone depolarised the bacterial inner membrane, with enhanced effects when combined with colistin at 1/8 MIC, a result not observed with colistin alone at this concentration. As with PI uptake, this inner membrane depolarising effect was more pronounced with myristic acid-loaded LNPs than with (E,E)-farnesol-loaded LNPs in resistant strains, suggesting that the colistin adjuvant effect of these lipophilic compounds is due to their ability to help colistin destabilise the bacterial inner membrane. Cytotoxicity assays demonstrated no adverse effects on bone marrow macrophages after 6 h of exposure, although some toxicity was observed after 24 h. No mortality was observed in *Galleria mellonella* larvae over 7 days following three consecutive days of treatment with colistin and LNPs. Notably, the combination of (E,E)-farnesol-loaded LNPs and colistin significantly improved the survival of *Galleria* infected with *A. baumannii*. These results suggest that lipophilic-adjuvant-loaded LNPs may offer a promising strategy to enhance colistin efficacy and combat antibiotic-resistant *A. baumannii* infections.

1. Introduction

Pneumonia, a significant cause of global mortality, claimed an estimated 700,000 lives in 2019 (WHO, 2022), disproportionately affecting vulnerable populations like children under five, adults over 65, and

individuals with underlying health conditions (Janssens and Krause, 2004; Tantawichien et al., 2022). Within this context, *Acinetobacter baumannii* has become a particularly concerning pathogen. This bacterium is one of the most commonly isolated from the respiratory systems of patients suffering from ventilator-associated pneumonia (VAP)

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worldwide (Erbay et al., 2003; Garnacho-Montero et al., 2005; Wood et al., 2002).

While traditional antimicrobial therapies such as carbapenems, colistin and combination antibiotic regimens remain options for *A. baumannii* pneumonia, their efficacy is threatened by the alarming increase in multi-resistant strains, particularly carbapenem-resistant *A. baumannii* (CRAB), presenting a significant challenge to its treatment (Fernández-Cuenca et al., 2003; Hu et al., 2007; Martínez-Trejo et al., 2022). This growing resistance situation justifies the inclusion of *A. baumannii* on the WHO's list of priority 1 critical pathogens for which there is an urgent need to develop new antibiotics (WHO, 2017; World Health Organization, 2024).

In critically ill patients with pneumonia caused by CRAB, colistin (CST), a polymyxin antibiotic with high nephrotoxicity, emerges as a last-resort antibiotic to combat this life-threatening infection. To optimize its concentration in the lungs and reduce renal toxicity, CST is mainly administered directly to the lungs by inhalation as an inactive prodrug called colistimethate sodium (CMS) (Boisson et al., 2014). However, this last hope for treatment is threatened by the emergence of CST-resistant strains of CRAB, adding another layer of complexity to this already difficult fight (Ilsan et al., 2021; Thet et al., 2020). In *A. baumannii*, most CST resistance is due to changes in lipooligosaccharides (LOS), the main component of the bacterial outer membranes, made of lipid A and polysaccharide chains. Overexpression of the operon *pmrCAB* (e.g., in *pmrB* mutants) results in increased amount of phosphoethanolamine to LOS, reducing susceptibility to polymyxins, with minimum inhibitory concentration (MIC) values typically ranging from 8 to 128 mg/L for CST (Arroyo et al., 2011). Alternatively, mutations in *lpxACD* can cause complete LOS loss, leading to significantly higher CST resistance (MIC > 128 mg/L) (Moffatt et al., 2010). The deactivation of *hns*, a gene that regulates LOS modification and phosphoethanolamine addition to lipid A, is another resistance mechanism (Deveson Lucas et al., 2018). These multiple mechanisms of resistance are increasing in prevalence worldwide, (Ilsan et al., 2021; Thet et al., 2020) rendering them virtually untreatable with conventional antibiotics. Hence, exploring new treatment options and maximizing CST efficacy is crucial to preserve its potency against multidrug-resistant *A. baumannii*.

Naturally occurring linear lipophilic compounds, like terpene alcohols and fatty acids, show promise as antibiotic adjuvants that target bacterial membranes. These compounds can penetrate and destabilize bacterial membranes, making bacteria more susceptible to polymyxins like CST (Fischer et al., 2012; Kostoulias et al., 2016; Valcourt et al., 2021) or polymyxin B (Brehm-Stecher and Johnson, 2003). For instance, farnesol, a sesquiterpene alcohol, disrupts the membranes of *A. baumannii*. As a result, combining farnesol (4–16 mg/L) with CST (0–8 mg/L) allowed to reduce the MIC values of 11 *A. baumannii* CST-resistant strains by 4- to 128-fold, whereas neither farnesol nor CST alone had any effect at these concentrations (Han et al., 2023). Furthermore, *in vivo* studies demonstrated that the bacterial count in mice treated with the CST-farnesol combination significantly decreased after 24 h. However, the poor water solubility of these lipophilic adjuvants poses a major challenge for their direct therapeutic application.

One promising approach to overcome this limitation is the encapsulation of these compounds in water-dispersible lipid nanoparticles (LNPs). These nanocarriers are oil droplets surrounded by a shell of surfactant molecules, which enables them to be stably dispersed in aqueous environments. By carefully selecting biocompatible triglycerides as the oil core and using low-toxicity non-ionic surfactants, LNPs can be safely administered to humans at high doses, even by inhalation (Miao et al., 2023). Moreover, research suggests that LNPs can readily fuse with bacterial membranes, further enhancing their effectiveness as delivery vehicles for lipidic adjuvant molecules (Hamouda and Baker, 2000; Hwang et al., 2013). This fusion potentially facilitates the integration of the lipid adjuvant in bacterial membranes, further increasing its potency against target bacteria.

In this study, the formulation and screening of various lipophilic-adjuvant-loaded LNPs identified two particularly effective combinations with free CST: one containing (E,E)-farnesol and another containing myristic acid. These formulations significantly enhanced CST's efficacy against several *A. baumannii* clinical isolates, exhibiting at least a 16-fold improvement *in vitro* compared to CST alone. Notably, no mortality was observed in *Galleria mellonella* larvae following three repeated administrations of these combinations. Furthermore, *in vivo* assessment of antimicrobial efficacy using *Galleria mellonella* infection models revealed a significant increase in larval survival with combinations of free CST and lipophilic-adjuvant-loaded LNPs, highlighting their promising potential as therapeutic adjuvants to CST.

2. Materials and methods

2.1. Chemicals and bacterial strains

Medium-chain triglycerides (Labrafac® WL 1349), composed of caprylic/capric acid triglycerides and used as the oil phase in the formulation, were provided by Gattefossé S.A. (France). Kolliphor HS15 (Polyethylene glycol (15)-hydroxystearate), myristic acid, farnesyl acetate, cation-supplemented Mueller Hinton broth (MHB), Mueller Hinton agar (MHA) and CST sulfate (lot: LRAC9149) were purchased from Sigma-Aldrich (France). Citronellol, farnesol, (E, E)-farnesol, geraniol, nerol, nerolidol, palmitic acid were sourced from TCI Europe (Belgium), while linalool was provided by Thermo Scientific (France) and Phytol by BLD-pharm (Germany). Bodipy-tocopherol was prepared as previously described (Wang et al., 2021). PBS was purchased from Dutscher (France), and agarose gel D5 from Euromedex (France). Five clinical isolates of *A. baumannii* were used in this study (Table 1).

2.2. Terpene/fatty acid-loaded LNP development and characterization

Terpene alcohol- or fatty acid-loaded LNPs were prepared without chemically modifying the compounds, using a modified spontaneous emulsification protocol (Attia et al., 2017). Briefly, Labrafac® was mixed with either a terpene alcohol or fatty acid adjuvant and the surfactant Kolliphor® HS15 (Fig. 1) at predetermined weight ratios (Table 2). The oil phase composition is expressed as the weight percentage of the adjuvant (wt.%) relative to the total weight of oil and adjuvant. Two surfactant-to-oil ratios (SORs) were used: 60 % (SOR = 60) and 40 % (SOR = 40), while maintaining a total formulation mass of 200 mg. The mixture was initially heated at 80 °C for 10 min and then centrifuged at 14100g for 10 min (MiniSpin plus®, Eppendorf). Then, the mixture was reheated to 80 °C while stirring at 1400 rpm for 10 min (Thermomixer), after which 800 µL of cold (20 °C) 0.01 M phosphate-buffered saline (PBS) was added. Blank LNPs, without adjuvants, were prepared as controls.

The intensity-weighted mean hydrodynamic size (Z-average) and polydispersity index (PDI) of LNPs were determined by dynamic light scattering (DLS) using a Zetasizer Nano (Malvern, France). Triplicate LNP suspensions were diluted 1:50 (v/v) in MilliQ water and analysed at 25 °C with a scattering angle of 173°. Each sample was measured 10 times, and data were analyzed by a cumulants-based method. Data are reported as the average size distribution and PDI.

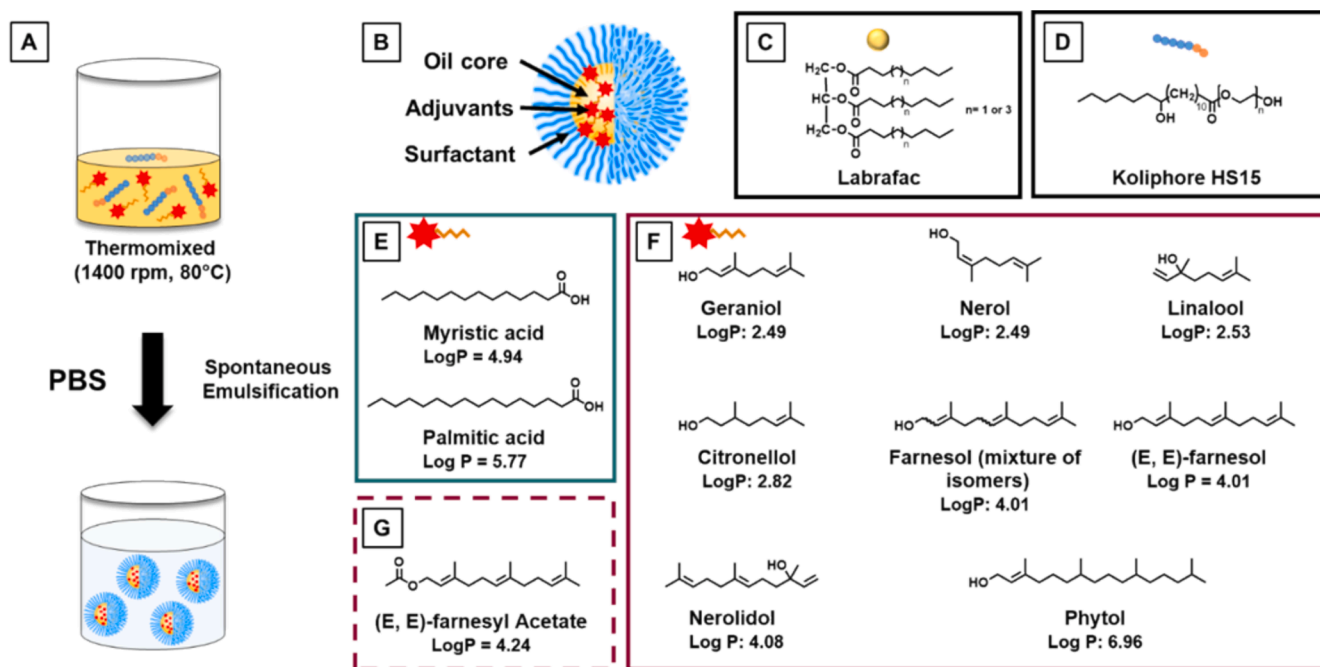
The zeta potential (mV) of unloaded and fatty acid-loaded LNPs was measured in triplicate using the same Zetasizer Nano equipped with a disposable Folded Capillary Zeta Cell (Malvern, France). Samples were diluted 1:20 (v/v) in 1 mM NaCl and subjected to three measurements each. Each measurement consisted of over 10 runs with an applied potential of 150 V.

The stability of the four most effective LNPs (in terms of CST efficacy enhancement) was monitored over time by measuring size and zeta potential at 0, 1, 2, 3, 6, 9, 16, 24, 45, and 92 days post-formulation. Triplicate samples were analyzed using the methods described above.

Table 1*Acinetobacter baumannii* clinical isolates: Descriptions, CST MICs (reported & experimental), tested concentrations, and sources.

Strains	Descriptions and references	Reported CST MIC value (mg/L)	CST MIC value in this study (mg/L)	CST concentration tested for MIC test (mg/L)
<i>A. baumannii</i> AbS1	Isolated in 2012 in the University Hospital of Sahloul (Sousse, Tunisia) from protected specimen brushes of the patient showing fever symptoms after 4 days of post-cardiac surgery procedure following intravenous ciprofloxacin, teicoplanin, and ceftazidime treatments for 11 days (Jaidane et al., 2018).	0.5	0.25–0.5	1–0.002
<i>A. baumannii</i> AbS2	Isolated from the same patient as AbS1, but from another protected specimen brushes and 2 weeks after antibiotic (CST, imipenem, amikacin, and rifampicin) treatments were discontinued (Jaidane et al., 2018).	>128	128–256	512–1
<i>A. baumannii</i> ABIsac_ColiS	Isolated in 2013 in Marseille, France from a bronchoalveolar lavage (BAL) specimen of a pneumonic French patient (Rolain et al., 2013).	N/A	0.25–0.5	2–0.004
<i>A. baumannii</i> ABIsac_ColiR	Isolated from the same patient as ABIsac_ColiS, but from the tracheal aspirate after 4 weeks of CST and rifampin treatments (Rolain et al., 2013).	N/A	4–8	16–0.031
<i>A. baumannii</i> Ab299	Isolated in 2008 from the bronchial secretions of a patient with a severe neurological disorder and respiratory distress who underwent tracheostomy and mechanical ventilation with ampicillin-sulbactam treatment (Pournaras et al., 2014).	0.5	0.5–1	2–0.004

N/A: CST MIC was not determined by the cited authors.

**Fig. 1.** Scheme of a lipid nanoparticle (LNP) and general procedure of LNP formulation (A–B). Skeletal formulae of the different LNP components: medium chain triglycerides oil (C), surfactant (D), and adjuvants: fatty acid (E), terpene alcohols (F), and terpene acetate ester (G).**Table 2**

LNP composition (wt.%) for two Surfactant-to-Oil Ratios (SORs), including adjuvants in the oil phase.

SOR (%)	Oil + adjuvant (%)	Surfactant (%)	Terpene alcohol or fatty acid (%)
60	40	60	12 or 4
40	60	40	18 or 6

2.3. Evaluation of electrostatic interaction of CST with fatty acid-loaded LNP by electrophoretic mobility shift

An electrophoretic migration assay was performed to evaluate the ion pairing between negatively charged fatty acid-loaded LNPs and positively charged CST. First, LNPs were formulated with 0.1 wt% of the fluorescent dye bodipy-tocopherol (BDP-Toco) (Wang et al., 2021) and varying amounts of palmitic and myristic acids (0.1–10 wt% of the oil

phase). Secondly, LNPs loaded with 10 wt% fatty acids were mixed with free CST in PBS at various negative-to-positive charge ratios (1:0–1:1). Charge ratios were calculated considering CST carries five positive charges, while each fatty acid carries one negative charge. Then, 12 μ L aliquots of 0.1 wt% BDP-Toco LNPs loaded with 10 wt% fatty acids were placed on a 0.5 wt% agarose gel in 1 \times tris-acetate-EDTA buffer. Electrophoresis was performed for 1 h at 125 V in the dark. Finally, the gels were imaged (Syngene™ Gbox Chemi XRQ, Syngene, USA) using excitation at 455–487 nm and emission detection between 516 to 539 nm. Each image acquisition employed a 10-second exposure time.

2.4. Minimum inhibitory concentration assay

Minimum inhibitory concentrations (MICs) of CST were determined in the presence of different concentrations of terpene alcohol or fatty acid loaded in LNPs using a microdilution method following EUCAST protocols (EUCAST, 2003). Briefly, 100 μ L of bacterial suspensions

standardized to 1×10^6 CFU/mL were added to 96-well plates containing 100 μ L of two-fold serial dilutions of CST (concentration range as per Table 1) and fixed concentrations of adjuvant loaded in LNPs.

Experiments were conducted in two settings. First, only LNPs loaded with various terpene alcohols prepared at 2 different sizes (SOR 40 % and SOR 60 %) were tested at 10, 50, and 100 mg/L in adjuvant against 3 clinical isolates (assays were repeated twice with 2 technical replications each). Second, LNPs of both sizes were loaded with (E, E)-farnesol, farnesol, farnesyl acetate, palmitic acid, or myristic acid and tested at 10, 60, and 120 mg/L against 5 isolates (Table 1). Tests were repeated 3–4 times with 2 technical replications each. All plates were incubated for 20 h at 37 °C and the MIC of CST required to inhibit bacterial growth (non-turbid) was determined. Due to variations in initial CST MIC for each strain (0.008–256 mg/L), results were expressed as relative CST MIC versus adjuvant concentration, with control (bacteria + CST alone) normalized to 1.

2.5. Time-kill curve analysis

Following protocols established in prior studies (Akroing et al., 2022; Valcourt et al., 2021), time-kill curves (TKC) were generated. Briefly, *A. baumannii* cultures grown overnight were diluted in MHB, incubated for an hour at 37 °C to reach logarithmic growth, and adjusted to 2×10^6 CFU/mL. TKC experiments were performed using LNPs formulated with a SOR of 60 % and adjuvant concentration of 60 mg/L, determined based on CST MIC results. To do this, 500 μ L of 4-times concentrated CST was transferred into wells of 24-well plate containing 500 μ L of 4-times concentrated LNPs loaded with either (E, E)-farnesol or myristic acid. Subsequently, 1 mL of *A. baumannii* AbS1 or AbS2 cultures (2×10^6 CFU/mL) was added into each well. The plates were incubated at 37 °C with agitation. Controls consisted of bacteria treated with CST or adjuvant loaded-LNPs alone. Aliquots of 100 μ L were collected at specific time points (0, 3, 6, 24, and 30 h), serially diluted in saline, and plated on MHA for colony counting (reported in Log₁₀ CFU/mL). These experiments were repeated 3–4 times.

2.6. Inner membrane permeability assay using propidium iodide

Propidium iodide (PI) uptake assays were performed to measure the effect of CST, alone or in combination with LNP (SOR 60 %), on plasma membrane permeability of *A. baumannii* isolates AbS1 and AbS2. After washing and adjusting the bacterial inoculum to 1×10^8 CFU/mL, a 4 \times concentrated CST solution was added into a black, 96-well plate (Greiner Bio-One, Austria) to achieve final concentrations of 0.25 mg/L (AbS1) and 16 mg/L (AbS2). Then, 4 \times concentrated LNPs loaded with (E, E)-farnesol or myristic acid were added for final concentrations of 60 and 120 mg/L each. The bacterial suspension (50 μ L) and 40 μ M PI (50 μ L) were then added to the wells. Fluorescence intensity (excitation λ = 560 nm, emission λ = 630 nm) was measured every 5 min for 60 min at 37 °C using an Infinite M200 Pro microplate reader (Tecan®, France). The assay was performed four times. PI fluorescence intensity (observation minus background) served as the outcome measure.

2.7. Inner membrane depolarization assay

The 3,3'-dipropylthiadicarbocyanine iodide (DiSC3(5)) assay was used to assess inner membrane depolarization induced by CST alone or combined with (E,E)-farnesol-loaded LNPs or myristic acid-loaded LNPs. Controls were made with pure PBS (pH 7.4) or LNPs alone. DiSC3(5) accumulates in cells with polarized membranes, where its fluorescence is quenched. Upon membrane depolarization, the dye is released, resulting in fluorescence emission (Elliott et al., 2020). Mid-log phase AbS1 and AbS2 were washed in PBS and adjusted to 10^8 CFU/mL. Bacteria were incubated with 0.5 μ M DiSC3(5) for 15 min, and then 4 \times concentrated CST and LNPs were added to a 96-well plate to achieve final concentrations of 0.25 mg/L CST (AbS1), 16 mg/L CST (AbS2), and

60 or 120 mg/L LNPs. Fluorescence intensity (excitation 622 nm, emission 670 nm) was measured every 5 min for 60 min at 37 °C using a Tecan® microplate reader. The assay was performed in triplicate, with DiSC3(5) fluorescence intensity (sample minus background) as the outcome measure.

2.8. Scanning electron microscopy (SEM) of bacterial surface

The combined effect of LNPs loaded with (E, E)-farnesol or myristic acid (SOR of 60 %) and CST on the morphology of *A. baumannii* isolate Abs2 was observed using SEM. Bacterial cultures were washed thrice in sterilized PBS, adjusted to 1×10^8 CFU/mL, and exposed for 1 h to CST (16 mg/L) alone or combined with 60 mg/L of (E, E)-farnesol or myristic acid loaded in LNPs. Bacteria were washed thrice with sterilized 0.9 % NaCl, fixed with 2.5 % glutaraldehyde 2 h (at 4 °C), dehydrated in ethanol (70 % for 2 min, 95 % for 15 min, and 100 % for 20 min, 25 °C), dried with hexamethyldisilazane (HMDS) (5 min at 25 °C), and coated with tungsten. Samples were observed using a TENE0 Volume Scope microscope (FEI, Hillsboro, OR, USA) under 10 kV.

2.9. Evaluation of the cytotoxicity of LNPs and CST on macrophages

Bone-marrow derived macrophages (BMDMs) were obtained from C57BL/6J mice as previously described (Henry et al., 2007). BMDMs were differentiated in DMEM medium (Invitrogen, France) supplemented with 10 % v/v FCS (Thermo Fisher Scientific, France), 10 % MCSF (L929 cell supernatant), 10 mM HEPES (Invitrogen, France) for 6 days. To assess cytotoxicity, a crystal violet (CV) assay was conducted. This assay involves extensive washing to eliminate non-adherent cells followed by staining the remaining (live) cells with CV. This assay was validated by visual inspection and by flow cytometry analyses using propidium iodide staining. Macrophages were seeded into 96-well plates at a density of 1×10^5 cells per well and allowed to adhere for 16 h before treatment with the indicated LNPs. At either 6 or 24 h post-treatment, cells were thoroughly washed 3 times with 200 μ L PBS and subsequently stained for 5 min with 50 μ L of CV solution (0.1 mg/mL in water). Cells were washed extensively to remove CV excess. Cell-associated dye was solubilized by adding 100 μ L of ethanol (70 % in water) and incubating the cells under light shaking for 5 min. The optical density was measured at 540 nm to calculate cell death. Normalization was performed using triton X100 (0.1 % v/v in water)-treated cells (100 % cell death) and untreated cells (0 % cell death).

2.10. Evaluation of the toxicity of LNPs and CST on *Galleria mellonella* larvae

In vivo toxicity of LNPs loaded with either (E, E)-farnesol or myristic acid (SOR 60 %) was assessed using *Galleria mellonella* larvae (Sud-Est Appats, France) survival. Unmelanized larvae (450–600 mg) were acclimated at 20 °C in the dark for 2 days upon arrival. Larvae were immobilized with forceps against a taped pipette tip (1000 μ L). A volume of 15 μ L was delivered to the left posterior proleg using an automated syringe pump (PHD 2000 Infusion, Harvard Apparatus, USA) equipped with a 1-mL plastic syringe (BD Plastipak™, France) and a 30 G needle (BD Microlance™ 3, France) connected by tubing. Larvae (10 per group) received PBS (control), CST (2.5 or 10 mg/kg), CST (2.5 or 10 mg/kg) with 75 mg/kg (E, E)-farnesol-loaded LNPs, or CST (2.5 or 10 mg/kg) with 75 mg/kg myristic acid-loaded LNPs (7 \times higher than CST based on TKC findings). Following injection, larvae were incubated at 37 °C in the dark and monitored daily for seven days. Mortality was defined as lack of response to touch and blackening. The experiment was repeated twice, and mean survival rates were calculated.

2.11. Efficacy assay in *Galleria mellonella* larvae

To establish a lethal infection (1×10^6 CFU/larvae), 10 larvae per

group were injected with 15 μ L of washed and diluted bacteria in PBS into the left posterior proleg. Treatment efficacy was assessed 30 min later by injecting the right proleg with 15 μ L of either PBS (control), CST (10 mg/kg), (E, E)-farnesol-loaded LNPs (75 mg/kg) with CST, or myristic acid-loaded LNPs (75 mg/kg) with CST. Larval mortality was monitored daily for seven days (37 $^{\circ}$ C, dark) across replicated experiments using fresh larvae (2 groups of 10 each). Survival between groups was compared using a log-rank (Mantel-Cox) test, with significance set at $p < 0.05$.

3. Results

3.1. Characterization of adjuvant-loaded LNPs

3.1.1. LNPs size distribution

Dynamic light scattering measurements were used to characterize the size (Z-average) and PDI of freshly prepared LNPs loaded with various adjuvants (terpene alcohols or fatty acids) (Fig. 2). LNPs prepared with both SORs exhibited monodispersed and homogeneous size distributions, but with significant size differences. LNPs prepared with a SOR of 40 wt% exhibited a size range of 120–158 nm and a mean PDI of 0.15 ± 0.03 (Fig. 2A). Conversely, LNPs formulated with a SOR of 60 wt% were smaller (32–69 nm) and had a lower PDI (0.12 ± 0.06) (Fig. 2B). Interestingly, the cargo type (fatty acids vs. terpene alcohols) did not influence LNP size despite differing loading percentages (10 wt% vs. 30 wt% oil phase). Long-term storage at 25 $^{\circ}$ C in water for 92 days resulted in minimal changes in size, PDI, and zeta potential, indicating excellent stability of the LNPs (Fig. S1 and S2). These findings demonstrate the ability to precisely control the size of LNPs through formulation parameters, in particular SOR. This precise control allows subsequent studies to explore how LNP size could modulate the adjuvant effect towards CST.

3.1.2. Evaluation of CST interactions with fatty acid-loaded LNPs

The loading of fatty acids had a significant impact on the surface charge of LNPs. With a constant SOR of 60 wt%, the absolute zeta potential of the LNPs increased proportionally with the increase in myristic or palmitic acid loading. Specifically, the zeta potential shifted from -1.8 ± 0.2 mV for blank particles to -8.8 ± 0.4 mV and -11 ± 1.0 mV for LNPs loaded with 10 wt% myristic or palmitic acid, respectively (Fig. 3 and Fig. S3). To explore how this fatty acid-induced negative

charge affected interactions with positively charged CST (Fig. 3A), LNPs loaded with 10 wt% fatty acids were combined with increasing concentrations of free CST, generating various molar charge ratios (from negative to neutral). The LNPs were then characterized for size, zeta potential, and electrophoretic mobility using agarose gel (Fig. 3 and Fig. S3). As CST concentration increased, both the zeta potential and the migration speed of the LNPs decreased. At a 1:1 M charge ratio, charge neutrality was achieved, and these values approached zero (Fig. 3D), indicating that CST interacts with the LNPs surface altering its electrical properties as depicted in Fig. 3B. Remarkably, the LNPs maintained stable in size throughout the experiment, indicating no aggregation, even after complete charge neutralization through CST adsorption (Fig. 3D).

3.2. In vitro microbiological evaluation

3.2.1. Effect of terpene alcohol-loaded LNPs on CST efficacy against *A. baumannii* clinical isolates

To assess the potential of terpene alcohol-loaded-LNPs to enhance CST efficacy, CST MICs were determined for *A. baumannii* isolates with varying CST susceptibilities in the presence of fixed terpene concentrations (10, 50, and 100 mg/L). Due to a broad MIC range (0.008–256 mg/L), results are presented as relative MICs normalized to controls with CST alone set to 1 (Fig. 4). Terpene alcohol-loaded LNPs alone had no effect on bacterial growth at the tested concentrations (data not shown). The data revealed a terpene concentration-dependent improvement of CST efficacy for most LNPs across all isolates, with potency linked to the specific terpene alcohol loaded (Fig. 4). Interestingly, LNP size (40 nm for 60 % SOR and 120 nm for 40 % SOR) did not influence this effect.

Among the tested terpenes, phytol (16-carbon backbone, most lipophilic) showed the least improvement, with only a modest increase in CST activity against the CST-susceptible isolate AbS1. Farnesol (12-carbon backbone) significantly enhanced CST efficacy, achieving up to a 64-fold reduction in the MIC at 50 mg/L, with generally no further improvement observed at higher concentrations. In contrast, nerolidol, which shares the same backbone length as farnesol but has a hydroxyl group at position C3 instead of C1, displayed lower efficiency against most isolates. Terpenes with shorter backbones (8-carbon backbone), such as geraniol, nerol, and citronellol, exhibited a weaker CST adjuvant effect than farnesol. Notably, geraniol and nerol, which are Z and E isomers, produced comparable effects on boosting CST efficacy. These

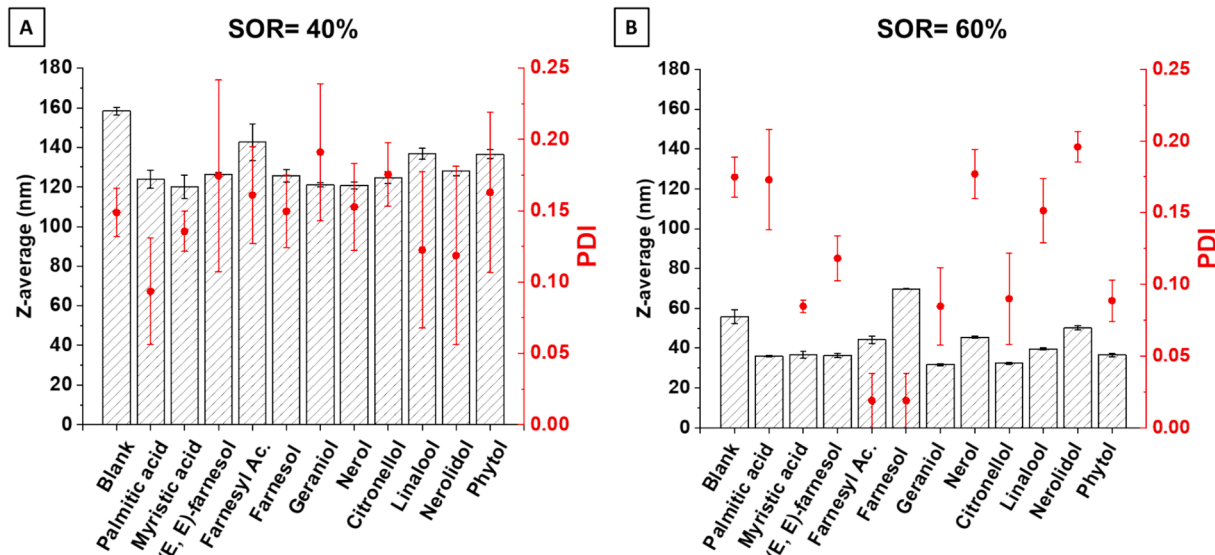


Fig. 2. Z-average particle size (bars) and polydispersity index (PDI, dots) of lipophilic-adjuvant-loaded lipid nanoparticles (LNPs), prepared with 10 wt% and 30 wt% oil phase loading for fatty acid and terpene, respectively. Two surfactant-to-oil ratios (SOR) were used: (A) 40 wt% and (B) 60 wt%. Data are presented as the mean \pm standard deviation of three independent replicates.

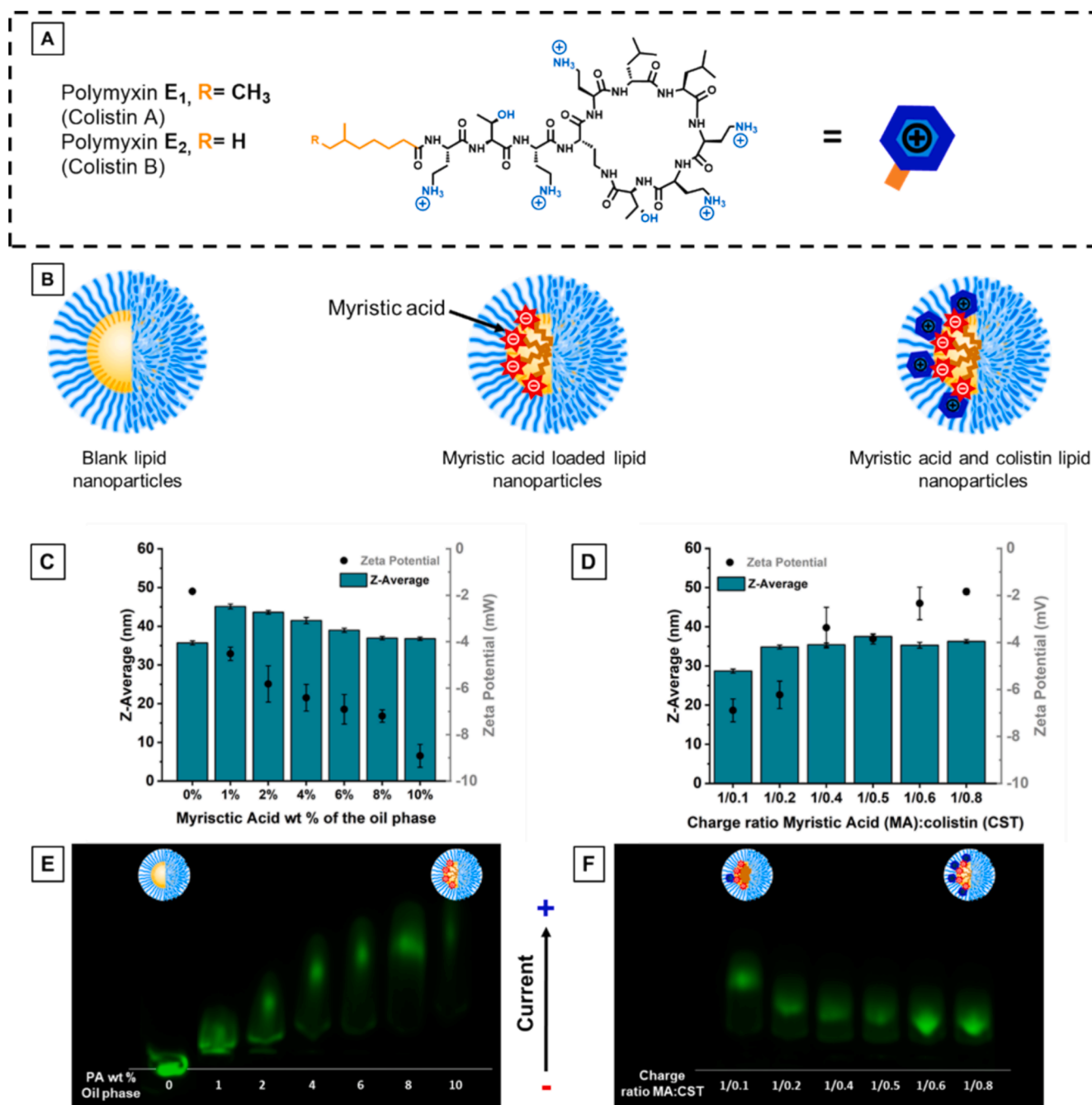


Fig. 3. Effect of fatty acids loading on LNPs interaction with CST. (A) CST chemical structure and its corresponding schematic representation. (B) Schematic representation of blank-, myristic acids loaded lipid nanoparticles (LNPs) and myristic acids loaded LNPs with electrostatic interaction with colistin. (C) Impact of myristic acid loading (0–10 wt%) on the size (Z-average – bars) and zeta potential (dots) of LNPs formulated with 60 wt% SOR. Effect of myristic acid/CST molar charge ratio on the size (bars) and zeta potential (dots) of LNPs loaded with 10 wt% palmitic acid. Data represent mean \pm standard deviation of three replicates. (E) Agarose gel electrophoresis analysis of LNPs loaded with 10 wt% myristic acid and bodipy-tocopherol (125 V for 1 h). (F) Agarose gel electrophoresis of LNPs loaded with 10 wt% myristic acid and bodipy-tocopherol in the presence of increasing CST concentrations to achieve various charge ratios.

data suggest that the length of the terpenol carbon backbone could be important in the colistin adjuvant effect.

Farnesol-loaded LNPs emerged as the most effective formulations, significantly enhancing CST activity against all tested *A. baumannii* isolates. Farnesol, a mixture of 4 diastereomers, was then further investigated to determine the individual contribution of the isomers. Thus, experiments employing a larger number of clinical isolates and replicates (3–4) were performed with farnesol-loaded LNPs and pure (E, E)-farnesol-loaded LNPs to evaluate the effect of a pure isomer compared to a mixture (Fig. 5). Additionally, the CST adjuvant effect of

LNPs loaded with farnesyl acetate, an acetate ester of farnesol with a comparable structure and logP value, but missing the hydroxyl group of the farnesol, was also tested. Interestingly, LNPs loaded with the mixture of farnesol's 4 diastereoisomers and LNPs loaded with pure (E, E)-farnesol behaved similarly. LNPs loaded with these compounds significantly reduced CST's MIC by up to 64-times from a concentration of 60 mg/L against 5 *A. baumannii* isolates, with no differences observed between LNPs formulated with a SOR of 60 % or 40 %. Conversely, LNPs loaded with farnesyl acetate were far less effective, only achieving a 4-fold CST MIC reduction at the highest concentration tested. These data

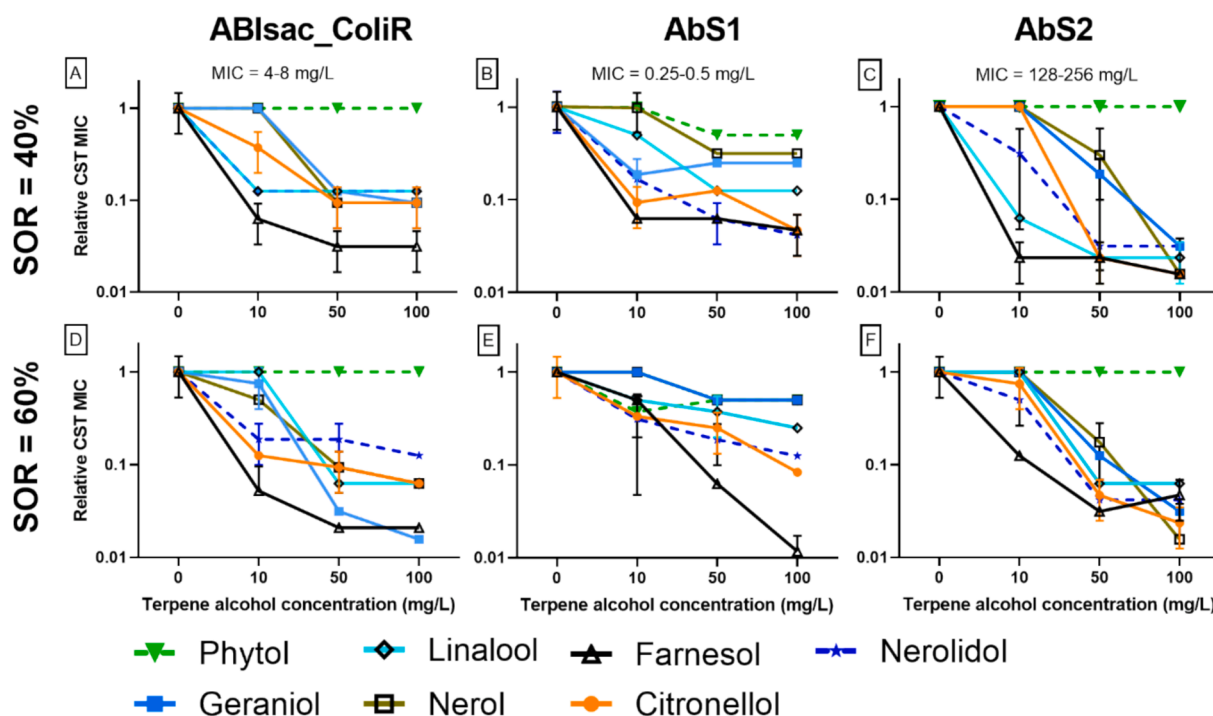


Fig. 4. Effect of terpene alcohol-loaded LNPs on relative CST MIC against three *Acinetobacter baumannii* isolates (ABIsac_ColiR, AbS1, and Abs2). Data for large (120 nm, 40 % SOR) and small (40 nm, 60 % SOR) LNPs are shown in upper and lower panels, respectively. Each data point represents the mean of all replicates (two independent experiments with two technical replicates each) \pm SD. CST MIC in the absence of adjuvant is indicated on the top of each panel.

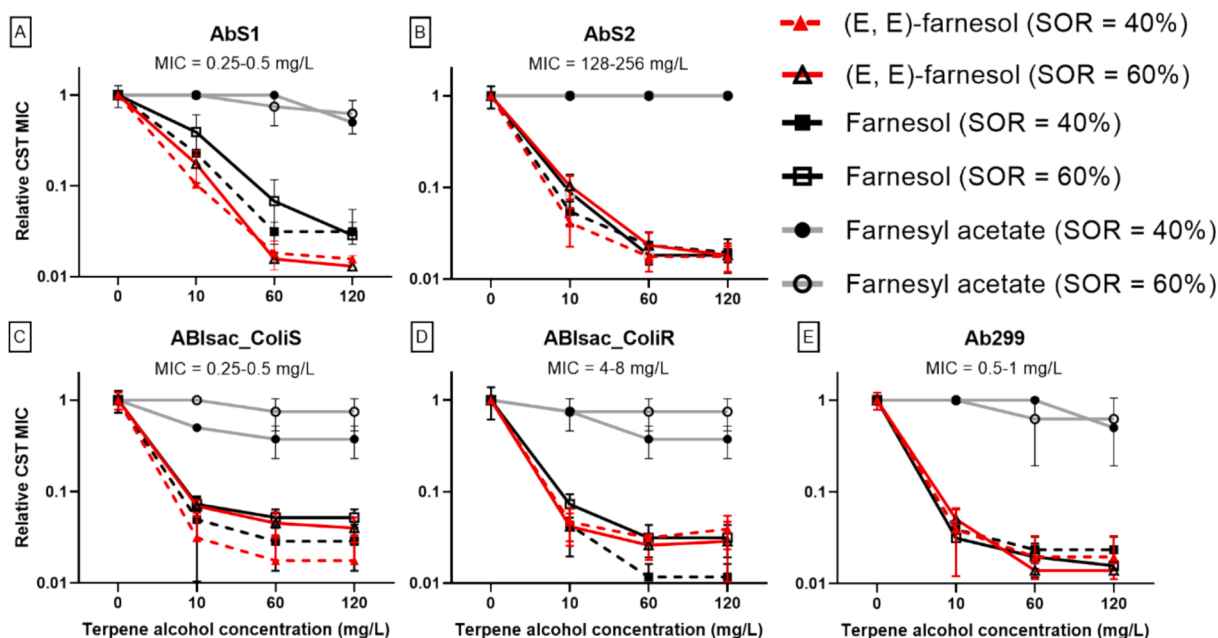


Fig. 5. Effect of farnesol vs. farnesyl-acetate loaded LNPs on relative CST MIC against five *Acinetobacter baumannii* isolates (AbS1, AbS2, Ab299, ABIsac_ColiS, and ABIsac_ColiR). Data (mean \pm SD from 3 to 4 replicates) represent the relative CST MIC as a function of the terpene concentrations.

suggest a potential role for the farnesol structure (hydroxyl group, length) in enhancing CST efficacy.

3.2.2. Effect of fatty acid-loaded LNPs on CST efficacy against *A. baumannii* clinical isolates

Similar to linear terpene alcohols, fatty acids share a linear hydrocarbon structure. This resemblance raises the possibility that, like terpenes, fatty acids might assist CST in disrupting bacterial membranes.

However, fatty acids diverge from terpenes in two key aspects: they lack the signature isoprenic unit and possess a carboxyl group instead of an alcohol group. These distinct functionalities could provide valuable insights into the chemical characteristics crucial for CST's adjuvant effect. To explore this possibility, the potential of LNPs loaded with two specific fatty acids, palmitic acid and myristic acid, to enhance the efficacy of CST against five clinical isolates of *A. baumannii* was investigated (Fig. 6). Similar to terpene alcohol-loaded LNPs, fatty acid-loaded LNPs

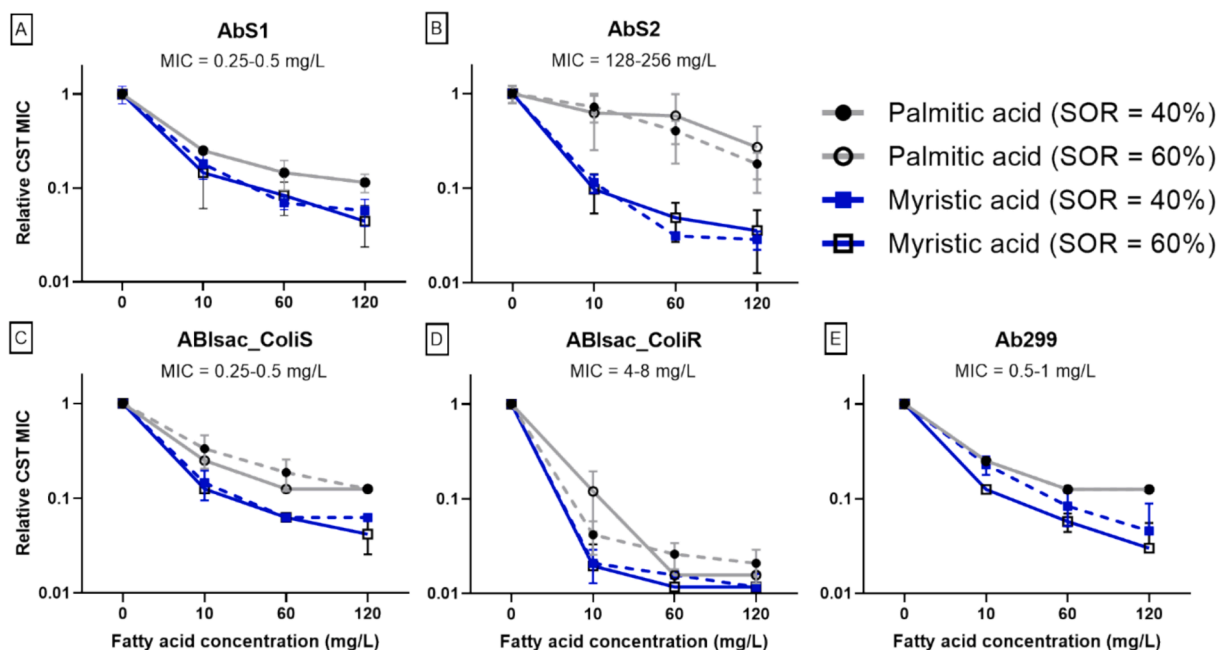


Fig. 6. Effect of fatty acids-loaded LNPs on relative CST MIC against five *Acinetobacter baumannii* isolates (AbS1, AbS2, Ab299, ABIsac_ColiS, and ABIsac_ColiR). Data (mean \pm SD from 3 to 4 replicates) represent the relative CST MIC as a function of the fatty acid concentrations.

alone had no impact on bacterial growth (data not shown) but boosted CST efficacy in a concentration-dependant way. Against most of the isolates, the improvement of CST efficacy was more gradual compared to what was obtained with farnesol-loaded LNPs. The type of fatty acid loaded in LNPs and the specific bacteria influenced how much CST efficacy improved. Palmitic acid (16-carbon chain) boosted CST's efficacy against multiple isolates, lowering the CST MIC by up to 32 times at a concentration of 120 mg/L. Myristic acid (14-carbon chain), however, consistently outperformed palmitic acid across all isolates.

3.2.3. Effect of CST combined with LNPs loaded with (E, E)-farnesol or myristic acid on bacterial growth kinetics

To assess the bactericidal effect of CST in combination with (E,E)-farnesol and myristic acid-loaded LNPs, time-kill curves (TKCs) were generated using two *A. baumannii* isogenic strains: AbS1 (CST-susceptible) (Fig. 7A) and AbS2 (CST-resistant) (Fig. 7B). CST alone (0.02–0.06 mg/L) displayed minimal activity against AbS1, with only a temporary suppression of bacterial growth observed at 0.25 mg/L (followed by regrowth to $\sim 10^8$ CFU/mL at 24 and 30 h). However,

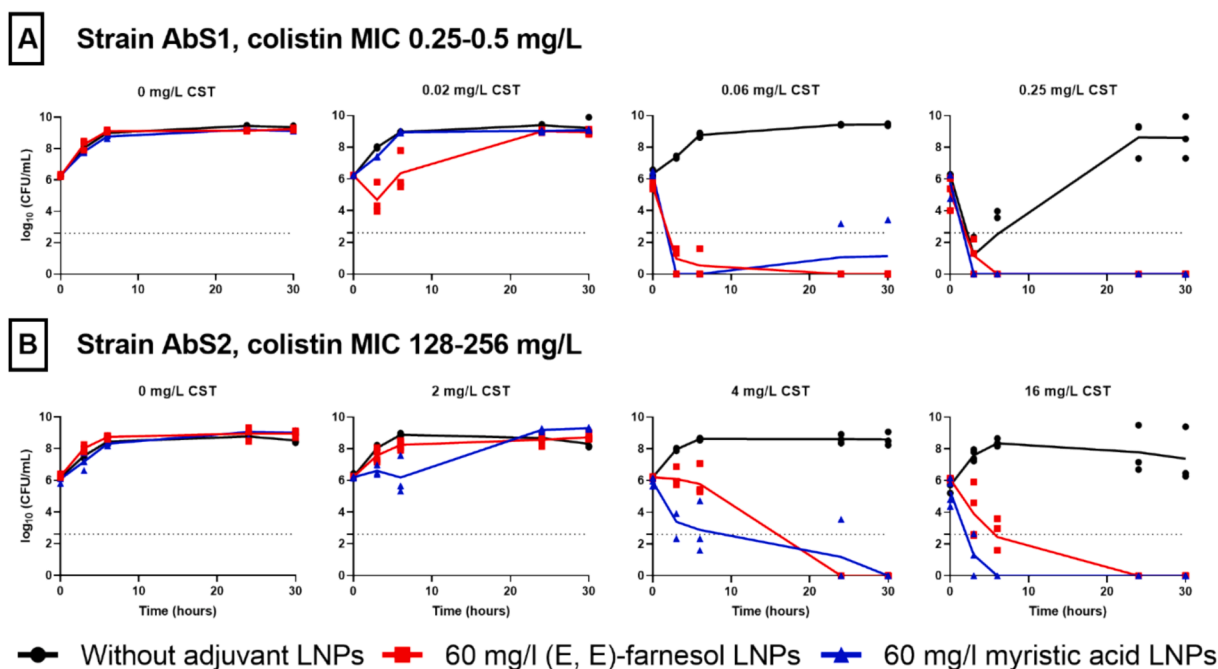


Fig. 7. Time-kill curve (TKC) assay of *Acinetobacter baumannii* clinical isolates AbS1 (A) and AbS2 (B). Each panel present TKC obtained for various CST concentrations, indicated on each panel, alone or in combination with either 60 mg/L of (E, E)-farnesol or myristic acid, both formulated as LNPs (SOR 60 %). Solid lines represent mean \log_{10} CFU/mL over time from 3 to 5 independent experiments (individual data points). The dotted line indicates the limit of quantification at 2.4 \log_{10} CFU/mL.

combining CST with 60 mg/L of either (E,E)-farnesol or myristic acid-loaded LNPs resulted in a significant bactericidal effect, reducing bacterial counts below the limit of quantification for all tested CST concentrations except 0.02 mg/L. Importantly, LNPs loaded with either compound alone (60 mg/L) had no effect on bacterial growth. Similar to Abs1, CST alone (2–16 mg/L) did not inhibit Abs2 growth. However, combining 16 or 4 mg/L CST with 60 mg/L of either LNP type resulted in bacterial killing with no regrowth at 24 and 30 h. However, 2 mg/L CST with either type of LNP did not inhibit Abs2 growth. Interestingly, the killing rate of Abs2 was faster with myristic acid-loaded LNPs compared to (E, E)-farnesol-loaded LNPs when combined with CST.

3.3. Effect of free CST combined with LNPs loaded with (E, E)-farnesol or myristic acid on bacterial membranes

Propidium iodide (PI) uptake analysis (Fig. 8A-B) as used to evaluate the impact of combining myristic acid- or (E,E)-farnesol-loaded LNPs with free colistin (CST) at 1/8 of its MIC on the integrity of the inner membrane in *A. baumannii* isolates Abs1 and Abs2. The isolates treated with the CST-LNP combination (CST MIC: 16 mg/L for Abs1 and 0.25 mg/L for Abs2) displayed a more rapid and significant increase in PI fluorescence compared to treatment with either CST or LNPs alone. Notably, PI fluorescence increased substantially after 10 min in Abs2 and after 25 min in Abs1 when exposed to the combined treatment. In contrast, neither CST nor the adjuvant-loaded LNPs (containing (E,E)-farnesol or myristic acid) induced significant PI uptake compared to the untreated control group. The extent of the PI fluorescence increase was influenced by both the bacterial isolate and the adjuvant used. For isolate Abs1, the combination of (E,E)-farnesol and CST resulted in a more pronounced increase in PI fluorescence than the myristic acid combination. However, for isolate Abs2, myristic acid induced a threefold higher increase in PI fluorescence compared to (E,E)-farnesol. Similar correlations were established for the mortality rates observed in the TKC (Fig. 7).

To further investigate the effects of the free CST-LNP combination on the inner bacterial membrane, a DiSC3(5) assay was performed to assess membrane depolarization (Fig. 8C-D). CST alone, at 1/8 of its MIC, did not induce depolarization in either isolate. However, myristic acid- or (E,E)-farnesol-loaded LNPs alone induced depolarization, the intensity of which depends on the concentration of the lipid adjuvant. When combined with free CST, the LNPs further enhanced membrane depolarization. SEM imaging of the Abs2 isolate revealed the development of wrinkled surface on the bacteria after 1 h of exposure to 16 mg/L CST (1/8 of MIC), though no cell lysis was observed (Fig. 8F). In contrast, Abs2 treated with 16 mg/L CST combined with 60 mg/L of either (E, E)-farnesol- or myristic acid-loaded LNPs caused severe surface damage with evident cell lysis, indicating disruption of both the outer and inner bacterial membranes (Fig. 8G-H).

3.4. In vitro and in vivo toxicity of (E, E)-farnesol or myristic acid-loaded LNPs combined with CST

The potential toxic effects of combining CST with (E, E)-farnesol or myristic acid-loaded LNPs were initially assessed *in vitro* using murine bone marrow macrophages. The cells were exposed to the formulations for 6 h (Fig. 9A) and 24 h (Fig. 9B). LNPs loaded with adjuvants, formulated at SOR 40 % and 60 %, were tested across a range of concentrations of (E, E)-farnesol or myristic acid (0–60 mg/L) combined with 0–16 mg/L of CST. After 6 h of incubation, no significant toxicity was observed at any concentration (Fig. 9A). However, after 24 h, cell death was evident at the highest adjuvant concentration (60 mg/L), regardless of the CST concentration. This cytotoxic effect was more pronounced with (E, E)-farnesol-loaded LNPs compared to myristic acid-loaded LNPs.

The potential *in vivo* toxicity of the free CST and LNP combination was further evaluated using *Galleria mellonella* larvae. Each larva

received daily administrations for three consecutive days, with a free CST dose of 10 mg/kg, consistent with previous studies in *G. mellonella* and human applications (Hill et al., 2014; Hornsey and Wareham, 2011). This dose was supplemented with 75 mg/kg of adjuvant-loaded LNPs formulated at SOR 60 %. After seven days of incubation at 37 °C, no larvae exhibited mortality or physical abnormalities, such as darkening or reduced mobility (Fig. 9C).

3.5. The combination of LNP loaded with lipid adjuvants and CST improves the survival of *G. mellonella* after infection with *A. baumannii*

The combined efficacy of CST with LNPs loaded with (E, E)-farnesol or myristic acid against Abs1 (Fig. 10A) and Abs2 (Fig. 10B) infections was investigated in *G. mellonella* larval model. Larvae were injected with 15 µL of 10⁸ CFU/mL to achieve ~10⁶ CFU/larvae followed 30 min after by treatment with 15 µL of the drug combination. Within 24 h post-infection, all larvae in challenged groups showed reduced mobility and darkening (Fig. 10C-F), with mortality starting. At this point, survival rates for larvae in control groups infected with Abs1 were 55–60 %, while those treated with CST + loaded LNPs reached 85–90 %. By day 3, control survival rates dropped to 10–55 %, whereas the groups treated with CST + LNPs remained 70–85 %. At the end of the observation period (7 days), the survival rate in the CST + lipid adjuvant-loaded LNPs groups (50 %) was significantly higher ($p \leq 0.0125$) than in the untreated control group (15 %). In contrast, none of the treatments significantly ($p \geq 0.05$) reduced mortality from Abs2 infection, even though survival rate of untreated and larvae treated with CST + (E, E)-farnesol LNPs were 10 % and 30 %, respectively.

4. Discussion

Terpene alcohols and fatty acids, known for their anti-inflammatory, antioxidant, analgesic, and antimicrobial properties (Kumar et al., 2020; Paduch et al., 2007) hold exciting promise for overcoming antibiotic resistance. Studies reveal that these compounds can potentiate the efficacy of antibiotics against various bacterial pathogens at concentrations below their MICs (Kumar et al., 2020). For instance, farnesol at 111 mg/L potentiates CST efficacy against multidrug-resistant *A. baumannii* isolates, enabling a 4–5 log₁₀ reduction in CFU/mL after 24 h, whereas CST alone at the same concentration (MIC) was almost ineffective (Kostoulias et al., 2016). Similarly, farnesol substantially reduces the MIC of CST (4–256 times) for multiple CST-resistant gram-negative bacteria, including *A. baumannii*, *K. pneumoniae*, *E. coli* (Han et al., 2023; Valcourt et al., 2021). However, time-kill studies suggest a more nuanced picture. While the combination initially suppresses bacterial growth, regrowth can occur later, raising concerns about long-term effectiveness. Interestingly, saturated fatty acids like caprylic (C8 acid), pelargonic (C9 acid), and capric (C10 acid), at relatively high concentrations (2 g/L), but below their MICs (1/4 MIC), can also potentiate CST efficacy (16–32 fold) against *E. coli* and *K. pneumoniae* (Cai et al., 2023). Notably, neither terpene alcohols nor fatty acids alone inhibited bacterial growth at these sub-optimal concentrations in these studies. This suggests their potential to act synergistically as antibiotic adjuvants.

4.1. (E, E)-farnesol or myristic acid were the most potent adjuvant to colistin

Despite challenges like low water solubility of terpene alcohols and fatty acids, our prior research showed promise for these compounds in enhancing free CST efficacy against *E. coli* when loaded into LNPs (Tewes et al., 2020; Valcourt et al., 2021). Inspired by this, we formulated LNPs containing different acyclic terpene alcohols or different saturated fatty acids at varying surfactant-to-oil ratios (SORs) using a spontaneous emulsification method allowing to formulate nanoparticles with sizes of 40 or 120 nm (Fig. 1). These 24 formulations were

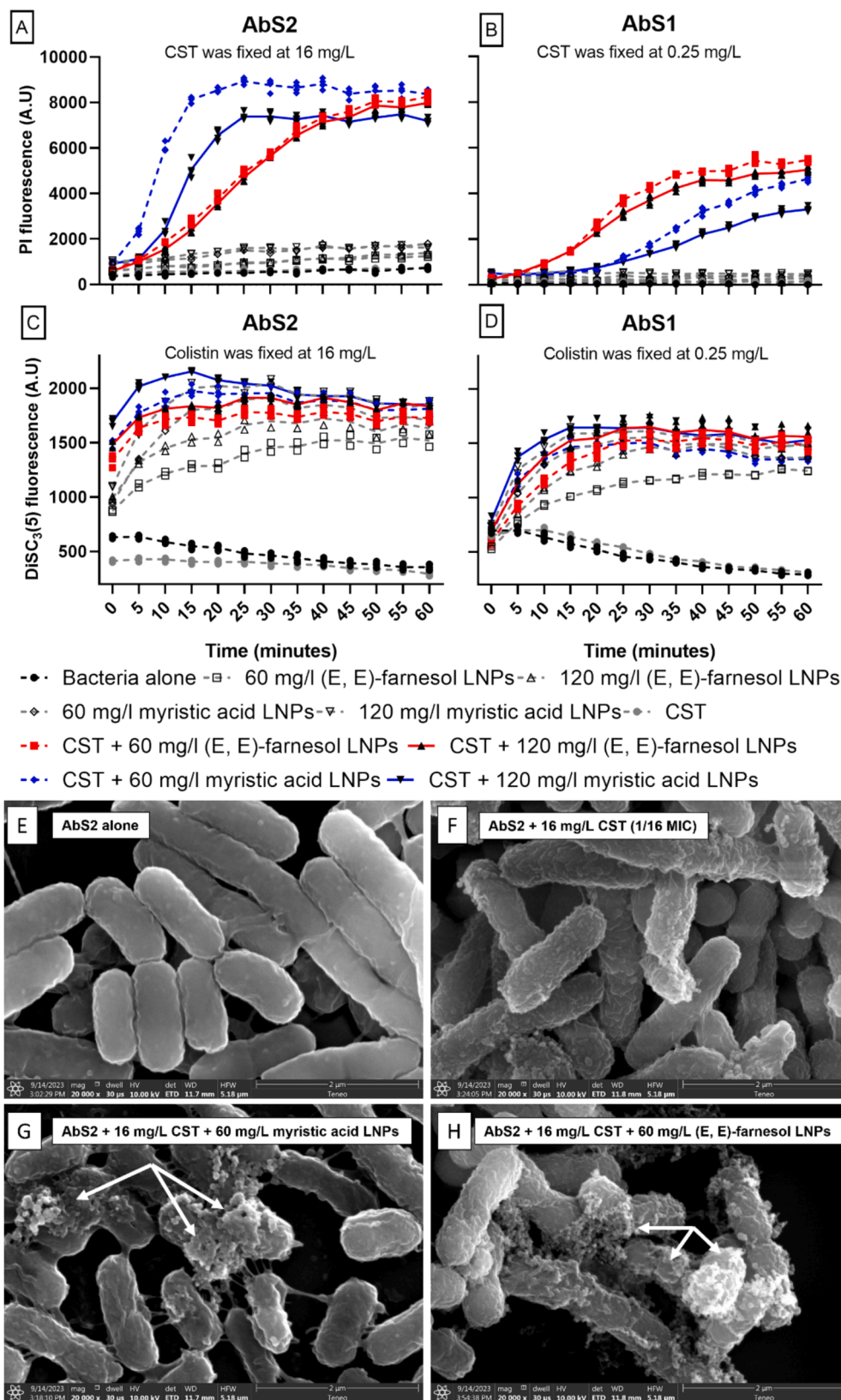


Fig. 8. Propidium iodide (PI) uptake, inner membrane depolarisation (DISC3(5) assay), and SEM observations of *Acinetobacter baumannii* clinical isolates after various treatments. (A-B) PI uptake by *A. baumannii* isolates AbS2 (A) and AbS1 (B) after 1 h of treatment. (C-D) DISC3(5) fluorescence in *A. baumannii* isolates AbS2 (A) and AbS1 (B) after 1 h of treatment. Treatments included CST alone, myristic acid-loaded LNPs, (E, E)-farnesol-loaded LNPs, and combinations of CST with each LNP type. Solid lines represent mean ± SD of 4 experiments. (E-H) SEM images of *A. baumannii* isolate AbS2. (E) Untreated control cells. (F) Cells treated with free CST (16 mg/L – 1/8 of MIC) for 1 h. (G & H) Treatment with CST (1/8 of MIC) combined with LNPs (visible cell lysis and indistinguishable cellular content are highlighted by white arrows). Scale bar = 2 μm.

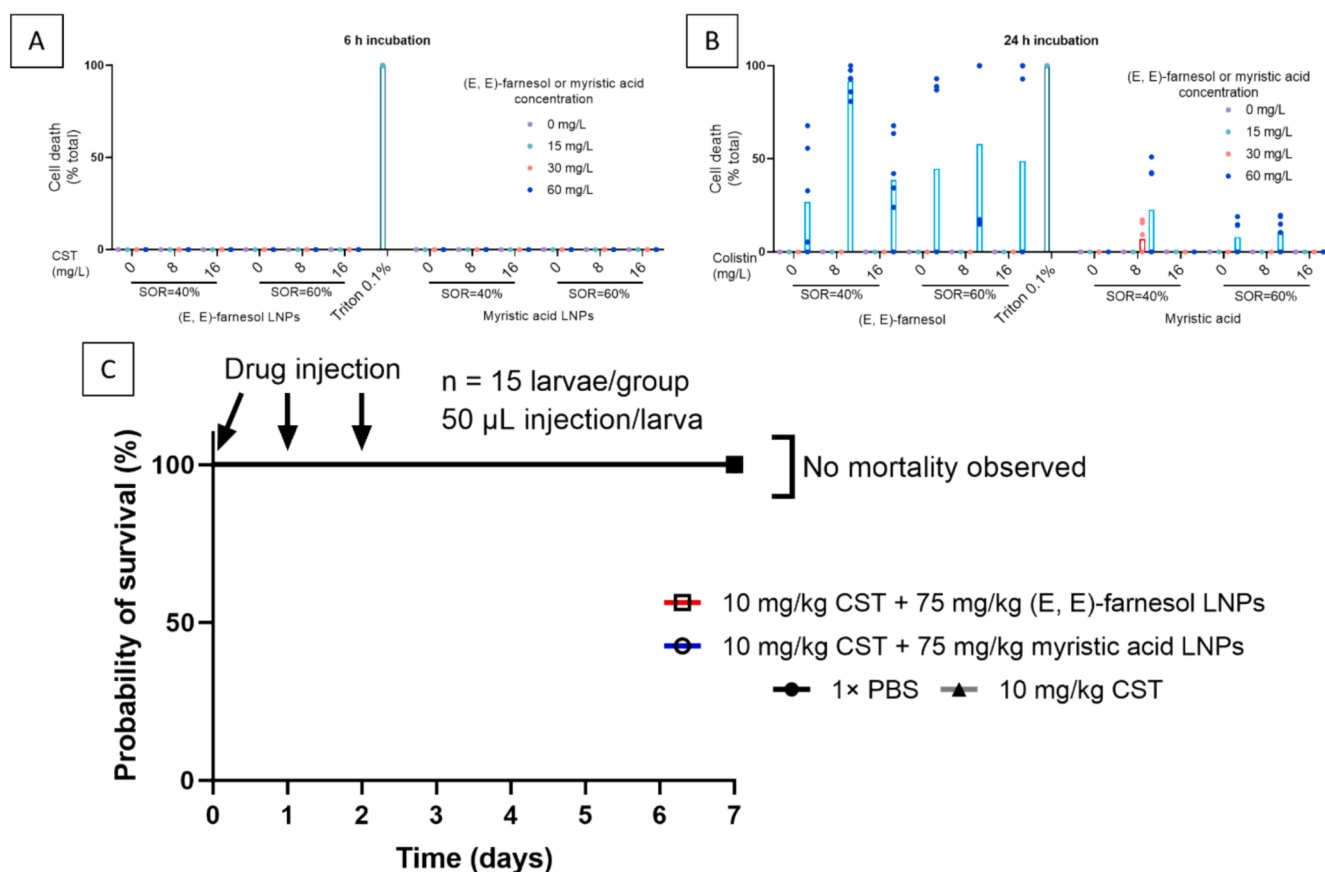


Fig. 9. *In vitro* and *in vivo* toxicity of adjuvant-loaded LNPs in combination with CST. Murine bone marrow macrophages were incubated for 6 h (A) or 24 h (B) with varying concentrations of (E, E)-farnesol or myristic acid (0–60 mg/L) formulated in LNPs (SOR 40 % and 60 %) in the presence of 0–16 mg/mL CST. Cell viability was assessed using the crystal violet assay, with each data point representing one well. Mean values (bars) are shown from two independent experiments performed in triplicate using two separate LNP preparations. (C). *Galleria mellonella* larvae survival over 7 days following three consecutive daily administrations of 10 mg/kg CST and 75 mg/kg lipophilic-adjuvant-loaded LNPs (SOR 60 %), a dose at least 7 times higher than CST, mirroring conditions from TKC studies. Solid lines represent the mean data from two independent experiments conducted at different times, with 10 larvae per group.

evaluated in combination with free CST against both CST-susceptible and -resistant *A. baumannii* isolated from patients with respiratory distress (Figs. 4–6). We discovered that at least half (6 out of 12) of the terpene alcohols and fatty acids incorporated into LNPs significantly reduced the CST MIC of our tested strains by 16–64 fold. Notably, LNPs containing either (E, E)-farnesol or myristic acid were the most potent in inhibiting bacterial growth in the initial hours, with no regrowth observed at later timepoints (30 h) when administered with free CST (Fig. 7). Interestingly, these same LNPs alone did not inhibit bacterial growth, solidifying their role as adjuvants without inherent antimicrobial activity. While LNPs containing (E,E)-farnesol or myristic acid showed cytotoxic effects on murine bone marrow macrophages after 24 h of exposure, no toxicity was observed after only 6 h. Furthermore, no mortality was recorded in *Galleria mellonella* larvae following daily administrations over three consecutive days with a combined dose of 10 mg/kg of free CST and 75 mg/kg of LNPs. A single administration of this combined dose significantly improved the survival rate of *A. baumannii* AbS1-infected larvae. The observed cytotoxicity is likely mitigated *in vivo*, as pharmacokinetic effects reduce prolonged exposure to high concentrations, and time-kill studies demonstrated that *A. baumannii* AbS1 (CST MIC ~ 0.5 mg/L) was eradicated within 3 h (Fig. 7A). Additionally, a separate study evaluating a similar combination of farnesol and CST at comparable concentrations found no cytotoxicity after 12 h of incubation with RAW264.7 macrophage-like cells (Han et al., 2023).

4.2. Terpenes and fatty acids disrupt bacterial membranes, enhancing colistin efficacy

Terpenes and fatty acids are known membrane spacers, inserting themselves near the polar head group region of membranes and increasing their fluidity, potentially compromising their function (Di and Kg, 1979; Mendanha and Alonso, 2015; Turina et al., 2006). Our study demonstrated that LNPs loaded with either farnesol or myristic acid, when combined with free CST, significantly increased inner membrane permeability in *A. baumannii* at concentrations as low as 1/8 of CST's MIC, a dose at which CST alone showed no effect (Fig. 8). Additionally, the DiSC3(5) assay confirmed that CST alone, at 1/8 of its MIC, failed to induce inner membrane depolarization in *A. baumannii* isolates. However, LNPs loaded with myristic acid or (E,E)-farnesol alone triggered membrane depolarization, which was further amplified when combined with CST at the same sub-MIC concentration (Fig. 8). This disruption of bacterial inner membrane ultimately led to bacterial death. Similar findings were reported by other studies. For example, it was found that farnesol alone (32 mg/L) slightly increased membrane permeability in *P. aeruginosa* TL2314 but was not bactericidal (Han et al., 2023). However, combining farnesol with CST (1–2 mg/L) resulted in pronounced PI uptake and bacterial death. This synergistic effect was also observed in another study with fatty acids (C8 to C10) and *E. coli* (Cai et al., 2023). In our study, LNPs loaded with lipophilic adjuvants alone caused only modest increases in PI fluorescence or membrane depolarization, without significant bactericidal activity. However, co-administration with CST resulted in substantial increases in

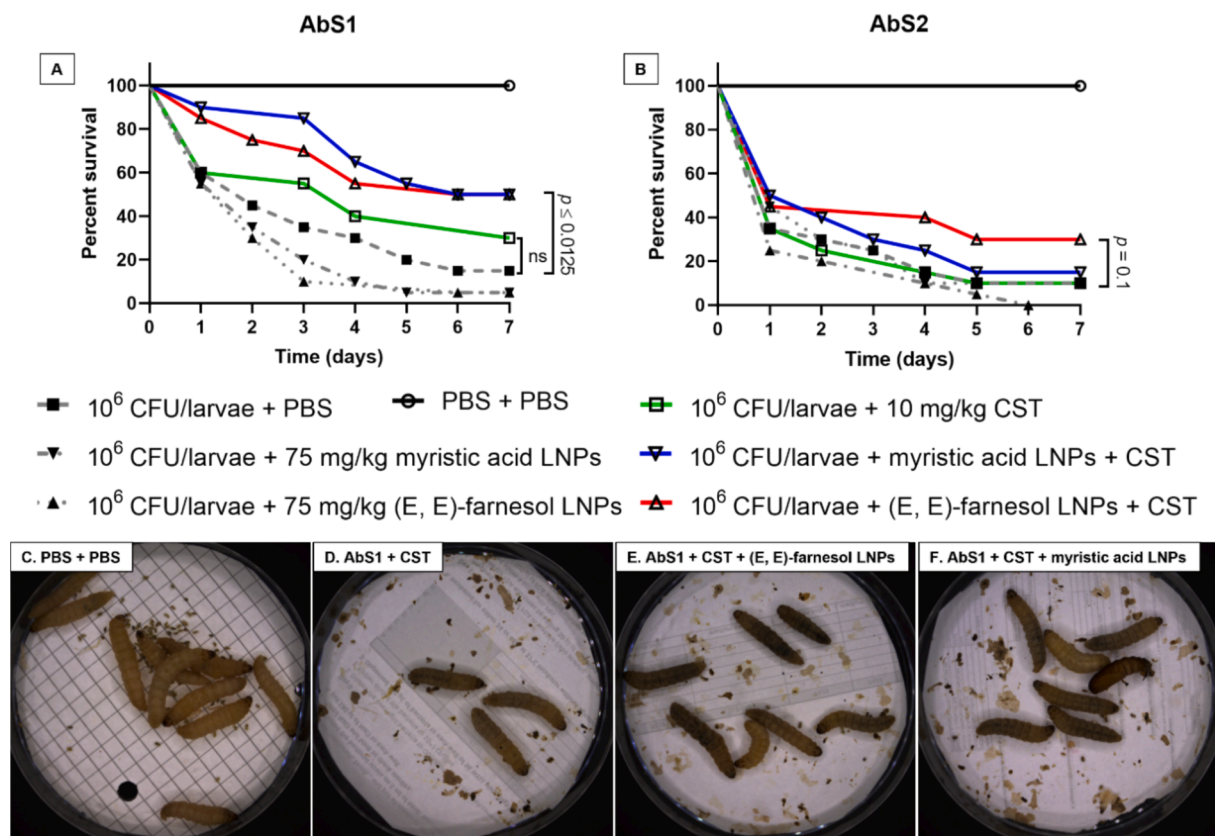


Fig. 10. Survival of *Galleria mellonella* larvae following infection with *A. baumannii* Abs1 (A) and Abs2 (B). Larvae were infected with 10^6 CFU/larvae. Solid lines represent mean of data from two independent experimental repetitions at two different occasions with 10 larvae/group/repetition. Uninfected larvae treated with PBS (PBS + PBS) appeared creamy white and highly mobile (C), while infected larvae presented dark coloration and reduced mobility at day 7 post-infection, as shown with the pictures obtained from one group infected with *A. baumannii* Abs1 (D, E, and F).

PI fluorescence, pronounced membrane depolarization, and bacterial death. These results, along with the findings from other studies, emphasize the potential of lipophilic compounds as adjuvants for enhancing CST efficacy. By co-destabilizing bacterial membranes, particularly the inner membrane, these compounds significantly improve the bactericidal effect of CST.

4.3. The structure of these lipophilic compounds seems to influence their effectiveness as CST adjuvants

The structure of farnesol, particularly the presence of the hydroxyl group, seems crucial for this effect. LNPs loaded with farnesyl acetate, where the hydroxyl group is capped by an acetyl group, were inefficient compared to farnesol. Similarly, previous studies showed that terpenes with alcohol groups exhibit better antimicrobial activity than those lacking them. For example, nerol and linalool were more than 10 times more active against *E. coli* than their esterified acetate counterparts (Griffin et al., 1999). Likewise, higher antimicrobial activity against *E. coli* and *Salmonella enterica* was observed for terpenols compared to hydrocarbonated terpenes, again linked to the presence of hydroxyl groups (Guimarães et al., 2019). Trombetta et al., showed that the antimicrobial effect of some terpene alcohols is partly due to disruption of bacterial membrane. They used large unilamellar vesicles (LUVs) as a model membrane and measured the release of a trapped dye in the presence of terpene alcohols and linalyl acetate. Terpene alcohols caused a significantly higher release (80–100 %) compared to linalyl acetate (30 %) (Trombetta et al., 2005).

However, we also observed that some terpene alcohols loaded within LNPs, such as phytol, citronellol, and linalool, did not perform well as CST adjuvants. Also, other factors like, molecular weight, octanol–water

partition coefficient (Log P), hydrogen bonding capacity, and unsaturation likely play a role. For instance, geraniol, nerol, linalool, and citronellol are all acyclic terpene alcohols. Yet, citronellol was inactive against *E. coli* compared to the others terpene (Griffin et al., 1999). The only structural difference is the absence of a double bond in citronellol. Our observations also suggest that molecules that are either too hydrophilic or too lipophilic have reduced CST adjuvant capacity. The overall trend seems to be: acyclic sesquiterpenoid (e.g., farnesol, $\log P = 4.01$) > mono-terpenoid (e.g., citronellol, $\log P = 2.82$) > di-terpenoid (e.g., phytol, $\log P = 6.96$).

Similar to terpenes, the effectiveness of fatty acids as CST adjuvants is influenced by their chemical structure and lipophilicity. Our study found that myristic acid ($\log P = 4.9$) with a lower Log P displayed greater activity compared to palmitic acid ($\log P = 5.8$). This aligns with other research demonstrating a sweet spot for fatty acid chain length, where a balance between hydrophobicity and functionality is crucial. Cai et al. reported enhanced CST effects against mcr-positive *E. coli* with fatty acids in the $\log P$ range of -3 to 2 (Cai et al., 2023). Likewise, Bergsson et al. observed that lauric acid ($\log P = 4.2$) was most effective in killing *Neisseria gonorrhoeae*, while fatty acids with shorter (caprylic acid, $\log P = 2.2$) or longer (myristic acid) chains were less efficient (Bergsson et al., 1999).

Overall, these results suggest that a balance between hydrophobicity and chemical functionality is key for the effectiveness of lipophilic compounds as CST adjuvants. While a certain level of hydrophobicity is necessary for interaction with the bacterial membrane, excessive lipophilicity might hinder its ability to interact with CST or penetrate the membrane effectively.

5. Conclusion and perspective

This study successfully developed LNPs loaded with specific lipophilic compounds to enhance colistin (CST) activity against drug-resistant *Acinetobacter baumannii*. LNPs containing farnesol or myristic acid significantly reduced the MIC of CST needed to inhibit bacterial growth, demonstrating a synergistic effect. These findings suggest promise for treating ventilator-associated pneumonia caused by multi-drug resistant *Acinetobacter baumannii*. Our results align with the growing interest in combining CST with adjuvants or nanoparticle-based approaches to combat resistant bacteria. This approach offers potential advantages like improved biofilm penetration and suitability for nebulization delivery in the lungs. Furthermore, the *in vitro* and *in vivo* toxicity results for our LNPs on eukaryotic cells and insect larvae are encouraging for continued development. However, further studies are needed to fully validate this approach. First, the synergistic effects of these combinations may vary depending on the specific bacterial strain. Our study was limited to a small number of clinical isolates, highlighting the need for further testing on a broader range of strains. Second, while invertebrate models offer valuable preliminary insights, definitive conclusions may require validation in mammalian models, given the potential for species-specific differences in drug response.

CRedit authorship contribution statement

Carla Faivre: Writing – original draft, Methodology, Formal analysis, Data curation. **Farras Daffa Imtiyaz:** Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Julien M. Buyck:** Writing – review & editing, Supervision, Conceptualization. **Sandrine Marchand:** Project administration, Funding acquisition. **Melissa Marcotte:** Methodology, Formal analysis. **Thomas Henry:** Writing – review & editing, Funding acquisition, Conceptualization. **Nicolas Anton:** Writing – review & editing, Funding acquisition, Conceptualization. **Mayeul Collot:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Frédéric Tewes:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Nicolas Anton reports financial support was provided by French National Research Agency. 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 material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijpharm.2024.124907>.

Data availability

Data will be made available on request.

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