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Effect of nanoemulsion loaded with macela (*Achyrocline satureioides*) on the ultrastructure of *Staphylococcus aureus* and the modulating activity of antibiotics

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Nanoformulations with herbal actives for treating bovine mastitis present an alternative for controlling bacterial infections in the emerging scenario of antimicrobial resistance. In this study, we investigated macela (*Achyrocline satureioides*) nanoemulsion (NE-ML), a formulation developed for the treatment of bovine mastitis (registered under Brazilian patent application BR 10 2021 008630 0), in the context of its bactericidal mechanism(s) of action and potential synergism with commercial antimicrobials. The effect of NE-ML on the integrity and cell permeability of *Staphylococcus aureus* was evaluated by measuring the electrical conductivity of bacterial suspensions exposed to different concentrations of NE-ML and by assessing the release of cellular constituents. Damage to bacterial ultrastructures was analyzed by transmission electron micrographs. The synergism of NE-ML with beta-lactam antibiotics and aminoglycosides was evaluated by the checkerboard test method against *S. aureus* (n = 6). The relative electrical conductivity of the bacterial solution gradually increased over time, reaching high values after exposure to 1xMIC (52.3%) and 2xMIC (75.34%) of NE-ML. Total proteins were detected in the bacterial suspensions exposed to NE-ML, increasing in concentration over exposure time (p < 0.05). Through bacterial micrographs, we observed that exposure to NE-ML (1xMIC) affected the integrity of the plasma membrane with invaginations in the cytosolic region and alterations in the cell wall. The increase in NE-ML concentration resulted in greater damage to the ultrastructure of *S. aureus* with changes in bacterial cell division patterns. When NE-ML was combined with the beta-lactam antimicrobials, the interaction was indifferent, indicating no modulation of antimicrobial resistance. In contrast, when combined with the aminoglycoside, a synergistic interaction did occur. These general findings suggest that the bactericidal action of NE-ML begins in the plasma membrane, causing alterations in its permeability and integrity, and extends to the cell wall, cytoplasm, and cell division. Although synergy was restricted to the aminoglycoside by destabilizing the bacterial cell membrane, this suggests that NE-ML can induce the entry of other actives, potentially reducing their

therapeutic doses. Understanding the mechanism of action of this new nanoformulation is certain to drive pharmacological advances, broaden the perspective of its *in vivo* use, and improve the treatment of bovine mastitis.

KEYWORDS

macela-nanoemulsion, drug delivery systems, herbal actives, antimicrobial, synergy, ultrastructural damage, bovine mastitis, sustainable dairy systems

1 Introduction

Current agricultural production models and food systems are considered inadequate and unsustainable to meet societal demands in the face of the emerging global climate crisis (Rosati et al., 2021). The widespread use of antibiotics, which is considered a major contributor to the development of antimicrobial resistance, is another significant point observed in conventional production systems (Bezner Kerr et al., 2023). It is estimated that global sales of antimicrobials for intensive animal production will increase by approximately 11.5% by 2030 (Tiseo et al., 2020). Agroecological and organic production systems, as alternatives to the hegemonic model, are practices that seek to strengthen the long-term resilience of food systems and can thus be considered a counterpoint to the alarming situation (Gamage et al., 2023; Bezner Kerr et al., 2023). However, the use of drugs and other inputs to treat animals in organic and agroecological production systems is limited. In Brazilian organic production systems (MAPA, 2021), only a small number of substances are approved for the treatment of various animal diseases, including bovine mastitis. These limitations for the treatment of bovine mastitis in these livestock systems represent a significant challenge for maintaining animal welfare and certification of organic livestock products in the country and globally.

Bovine mastitis is a multifactorial disease affecting dairy cattle, characterized by inflammation of the bovine mammary gland (Ezzat Alnakip et al., 2014) and responsible for significant economic losses in the production sector (Hogeveen et al., 2011; Javed et al., 2022). *Staphylococcus aureus* is the main pathogen associated with cases of chronic and subclinical mastitis and is considered a contagious etiological agent (Fitzgerald, 2012), highly adaptive, opportunistic, and easily spread among animals during milking (Campos et al., 2022). It is also a facultative intracellular pathogen (Johns et al., 2015) that protects bacteria from defenses of the host immune system and from the action of low-penetration antibiotics in eukaryotic cells (Algharib et al., 2020). It can form biofilms (Vestby et al., 2020) that limit susceptibility to antimicrobial therapy due to low antibiotic diffusion (Amorena et al., 1999).

Antibiotic therapy is the main therapeutic approach in the management of intramammary infections in cattle (El-Sayed and Kamel, 2021). The cure rate for cases of subclinical mastitis during lactation can vary significantly, from 5% to more than 80% (Salat et al., 2008). Due to this variability, new treatment modalities, such as phage therapy (Varela-Ortiz et al., 2018), phage endolysins (Fenton et al., 2013), vaccines (Rainard et al., 2021), probiotic approaches (K. Wallis et al., 2018), stem cells (Singh et al., 2020), herbal medicines (Fiordalisi et al., 2018; Chen et al., 2019) and nanoparticles (Pinheiro Machado et al., 2019) have already been

investigated and described as potential tools for the control of staphylococcal bovine mastitis.

The use of phytochemicals as therapeutic molecules via the intramammary route has become a promising biomimetic approach for bovine mastitis management, serving as a potential alternative to the use of antibiotics (Touza-Otero et al., 2024). The development of new antimicrobial agents that incorporate nanosizing the composition of therapeutic actives allows these formulations to become more efficient based on their ability to cross different barriers of the organism, their high stability and bioavailability, as well as their sustained release and lower toxicity (Harwansh et al., 2019; Youssef et al., 2019). These properties can alleviate some of the problems associated with low bacteriological cure rates of bovine mastitis, which result from low penetration and intracellular retention of antibiotics and non-diffusion of acidic antibiotics across the lysosomal membrane at extracellular or neutral cytoplasmic pH (Gruet et al., 2001).

In this sense, a nanoemulsion containing macela extract (*Achyrocline satureioides*) was developed (Machado et al., 2020; Machado, 2021) as an alternative product for controlling bovine mastitis in organic and agroecological production systems (registered under Brazilian patent application BR 10 2021 008630 0). Macela (*A. satureioides* Lam. DC.), family Asteraceae (Compositae), is a medicinal herb native to South America (Lorenzo et al., 2000; Ruffa et al., 2002), and its biological activity is related to the presence of flavonoids (Simões et al., 1988; Petrovick et al., 2010; Joray et al., 2013; Moresco et al., 2017; Souza et al., 2018; Bianchi et al., 2019; Martínez-Busi et al., 2019).

Previous studies on the development of the macela-nanoemulsion showed an average particle size of around 200 nm, monodisperse distribution (narrow size distribution) (PDI<0.2), encapsulation efficiency of the extract compounds in nanoparticles greater than 94%, and physical stability over 160 days at room temperature (Machado et al., 2020). It is characterized as a sustained release system for bioactive compounds with antimicrobial activity. Macela-nanoemulsion showed high activity against *S. aureus* with a minimum inhibitory concentration (MIC) of 125 µg/mL of crude extract, corresponding to 5% of the nanoemulsion (v/v). By showing a cytoprotective effect for bovine mammary epithelial cells (MAC-T) and high penetration capacity into the mammary gland in a Franz cell model (Pinheiro Machado et al., 2022), NE-ML was incorporated into nanocomposites based on natural polymers (flaxseed mucilage and k-carrageenan) for the development of an antimicrobial hydrogel aimed at intramammary therapy of bovine mastitis. During the process of developing and characterization of the nanoemulsion loaded with macela extract (*A. satureioides*) (Machado et al., 2020; Machado, 2021; Machado et al., 2023), different formulations were tested to identify the most efficient

composition in terms of physicochemical stability and antimicrobial activity. Detailed information on the characterization of this nanoformulation can be found in [Supplementary Material](#).

In this article, we investigate the nanoemulsion loaded with macela extract (*A. satureioides*) (NE-ML) in the context of elucidating the mechanism of bactericidal action against *S. aureus* and its potential for modulating resistance to beta-lactam and aminoglycoside antimicrobials. Within the permitted pharmacological options and the requirements of organic and agroecological production systems, this formulation is considered a promising candidate bactericide against bovine mastitis.

2 Material and methods

2.1 Bacterial strains and antibiotics

The bacterial strains were the standard strain *S. aureus* ATCC 25923 and *S. aureus* isolated from milk of animals with subclinical bovine mastitis ([Kuhnen et al., 2021](#)) abbreviated O1, P1, P2, G1, and G2. Antimicrobial susceptibility of the strains was tested against oxacillin (oxacillin sodium - Novafarma Indústria Farmacêutica Ltda), benzetacil (benzathine benzylpenicillin - Eurofarma Laboratórios S.A.) and Gentatec[®] Mastite (gentamicin - Chemitec).

2.2 Preparation of macela-nanoemulsion

The macela extract was obtained from macerating the plant inflorescences in 80% ethanol (1:60, w/v), and the product was vacuum-filtered. The solvent was then removed in a rotary evaporator according to [Machado et al. \(2020\)](#). The aqueous phase of the crude extract was used to prepare a pre-emulsion containing golden flaxseed oil (*Linum usitatissimum* L.) and polysorbate 80 (Tween 80). The nanoemulsion was prepared using a high-pressure homogenizer (Homolab A10, Alitec, Brazil) following protocols developed by [Machado \(2021\)](#).

2.3 Antimicrobial activity of macela-nanoemulsion

The antimicrobial activity of NE-ML against *S. aureus* ATCC 25923 was evaluated using the broth microdilution method ([CLSI, 2018](#)) with modifications. This method allowed establishing the minimum inhibitory concentration (MIC), i.e., the lowest concentration of the product capable of reducing bacterial growth by 80% or more. The concentration range tested for NE-ML ranged from 40% to 0.31% (v/v), equivalent to 1,000 to 7.8 µg/mL of nanoencapsulated macela extract. The final bacterial inoculum density corresponded to 10⁵ CFU/mL⁻¹. Positive controls for bacterial growth and sterility of NE-ML, and Mueller Hinton Broth (MHB) were included. The microplate was incubated at a temperature of 37°C for 18–24 h in an incubator (SL-100 Solab). The MIC was visually confirmed by adding resazurin dye (100 µg/mL⁻¹) ([Sarker et al., 2007](#)). The assays were performed in triplicate.

2.4 Electrical conductivity of bacterial suspensions

Alteration of bacterial membrane permeability by NE-ML was evaluated through the electrical conductivity of the bacterial membrane ([Diao et al., 2014](#)). A bacterial suspension of *S. aureus* ATCC 25923 of approximately 1.8–2.5 × 10⁷ CFU/mL⁻¹ was centrifuged (High-Speed Centrifuge 5430 R, Eppendorf). The bacterial pellet was washed with 5% glucose until its electrical conductivity was equivalent to that of a pure 5% glucose solution, becoming isotonic. Isotonic bacteria were exposed to NE-ML concentrations of 1xMIC and 2xMIC (hereinafter referred to as L1 and L2, respectively), and the control group of isotonic bacteria without NE-ML (L0) was treated with boiling water for 5 min. 5% glucose solutions were treated with the addition of NE-ML at concentrations of 1xMIC and 2xMIC. The L0, L1, and L2 treatments were incubated at a temperature of 37°C, and their electrical conductivities were measured with a digital conductivity meter (Gehaka CG 1800) every 30 min over 3 h. Bacterial membrane permeability was expressed as Relative Electrical Conductivity (%) and calculated as (%) = 100 × (L2 – L1)/L0 ([Diao et al., 2014](#)). The assays were performed in triplicate.

2.5 Integrity of cell membrane

The integrity of the bacterial cell membrane was assessed by UV-VIS spectrophotometry. An initial screening allowed the detection of macromolecules, such as nucleic acids and proteins, released in *S. aureus* ATCC 25923 suspensions treated with NE-ML (adapted from [Diao et al., 2014](#)). Bacterial suspensions of approximately 1.8–2.5 × 10⁷ CFU/mL⁻¹ were centrifuged (High-Speed Centrifuge 5430 R, Eppendorf), the supernatant discarded, and the pellet resuspended in 0.1 M phosphate-buffered saline (PBS buffer). The 1xMIC and 2xMIC concentrations of NE-ML were added to these suspensions. A bacterial solution in 0.1 M PBS buffer without NE-ML was used as control (C). The treatments were incubated at a temperature of 37°C, and after 2 and 4 h, aliquots were collected and centrifuged, followed by evaluation of the supernatant in a UV-VIS spectrophotometer (Bel UV-M51) at 260 and 280 nm. After finding the absorbent material, to determine the concentration of total proteins in the suspensions, new bacterial suspensions were prepared under the same incubation conditions in 0.1 M PBS buffer, with incubation times of 2 and 4 h and a temperature of 37°C both for the control suspensions without addition of NE-ML and for the treatments with the addition of 1xMIC and 2xMIC concentrations of NE-ML. Using both Bradford assay ([Bradford, 1976](#)) by incubation in a microplate reader and Coomassie Brilliant Blue BG-250 dye at 595 nm in a UV-VIS spectrophotometer, the concentration of total proteins was determined. The standard curve was determined from a bovine serum albumin (BSA) solution (1 mg/mL⁻¹). The assay was performed in triplicate.

2.6 Effect on bacterial ultrastructure

Alterations in the ultrastructure of *S. aureus* ATCC 25923 after exposure to NE-ML were confirmed through transmission electron microscopy (TEM). Concentrations of 1xMIC and 2xMIC of NE-

TABLE 1 MIC, antimicrobial sensitivity profile (CLSI, 2019), and concentration range of antimicrobials used in the checkerboard test against *Staphylococcus aureus* strains.

<i>S. aureus</i>	Atb	MIC ($\mu\text{g/mL}$)	Equivalent MIC Breakpoint ($\mu\text{g/mL}$)		Concentration range	
					NE-ML ($\mu\text{g/mL}$)*	Atb ($\mu\text{g/mL}$)
ATCC 25923	OXA	0.25 $\mu\text{g/mL}$	≤ 2	Susceptible	750 to 7.8	4 to 0.031
O1	OXA	0.25 $\mu\text{g/mL}$	≤ 2	Susceptible	750 to 7.8	4 to 0.031
P1	PEN	0.5 $\mu\text{g/mL}$	≥ 0.12	Resistant	750 to 7.8	4 to 0.031
P2	PEN	0.25 $\mu\text{g/mL}$	≥ 0.12	Resistant	750 to 7.8	4 to 0.031
G1	GEN	0.5 $\mu\text{g/mL}$	≤ 4	Susceptible	750 to 7.8	8 to 0.062
G2	GEN	1 $\mu\text{g/mL}$	≤ 4	Susceptible	750 to 7.8	4 to 0.031

Atb: antibiotic; MIC: minimum inhibitory concentration; *Values in $\mu\text{g/mL}$ refer to the concentration of nanoencapsulated macela extract from NE-ML, equivalent to 40% to 0.31% of NE-ML (v/v).

ML were added to bacterial suspensions ($1.8\text{--}2.5 \times 10^7$ CFU/mL⁻¹) (adapted from Diao et al., 2014), and a bacterial suspension without NE-ML addition was used as a control group. The treatments were incubated at 37°C, and after 2 and 4 h, aliquots were centrifuged (High-Speed Centrifuge 5430 R, Eppendorf), followed by washing the bacterial pellet with 0.1 M PBS buffer. The pellets were fixed with Karnovsky solution and then washed with 0.1 M Sodium Cacodylate Buffer (SCB). Post-fixation was carried out with a solution composed of osmium tetroxide (1%), potassium ferrocyanide (8%), and calcium chloride (5 mM) in 0.1 M Sodium Cacodylate Buffer (SCB), followed by another wash with 0.1 M SCB. Dehydration of the samples was carried out with increasing concentrations of acetone (30%, 50%, 70%, 90%, and 100%), and infiltration into epoxy resin occurred slowly (Embed-812, Electron Microscopy Sciences). The complete polymerization of the samples occurred at a temperature of 60°C for 48–72 h. The samples were cut using an ultramicrotome and contrasted with 2% uranyl acetate and lead citrate for subsequent analysis in a transmission electron microscope (TEM 100 kV - JEM-1011) (de Souza, 2011; Pergo et al., 2019). The assays were performed in triplicate.

2.7 Modulation of antimicrobial resistance

The checkerboard test allowed the evaluation of the modulatory resistance capacity of NE-ML associated with commercial antibiotics (Humphries, 2016). Initially, the MIC of NE-ML and beta-lactam antimicrobials (oxacillin and penicillin) and aminoglycosides (gentamicin) was determined using the broth microdilution method (CLSI, 2018). *Staphylococcus aureus* ATCC 25923 and strains O1, P1, P2, G1, AND G2 were selected based on their antimicrobial resistance profile (Kuhnen et al., 2021). Following the determination of the MIC of NE-ML and commercial antimicrobials for *S. aureus* strains, their concentration ranges were determined for the assay (Table 1).

For the checkerboard test (Humphries, 2016), a 96-well microplate was used, allowing different dilutions of two antimicrobials simultaneously (Bonapace et al., 2002; Orhan et al., 2005). The highest concentrations of each product were positioned in opposite corners of the microplate. The final bacterial inoculum corresponded to a bacterial density of

10^5 CFU/mL⁻¹. The microplate was incubated at 37°C for 18–24 h (Bonapace et al., 2002; Orhan et al., 2005). The addition of resazurin dye visually confirmed bacterial growth, allowing for result interpretation based on the bacterial growth and growth inhibition interface (Sarker et al., 2007). The assay was performed in duplicate.

The checkerboard assays resulted in a ΣFIC index (fractional inhibitory concentration index) (Humphries, 2016) per well tested on the microplate and calculated by comparing the MICs of the agents used in combination with the MICs of these agents alone. Therefore, $\Sigma\text{FIC} = \text{FIC}_A + \text{FIC}_B$, where $\text{FIC}_A = \text{MIC of agent A in combination}/\text{MIC of agent A alone}$, and $\text{FIC}_B = \text{MIC of agent B in combination}/\text{MIC of agent B alone}$. Each well tested in the microplate resulted in a ΣFIC index. After determining the bacterial growth and non-growth interface, the well with the lowest ΣFIC index among all wells without bacterial growth at the interface was used to determine the modulatory result as synergistic (≤ 0.5), indifferent ($>0.5 \leq 4$) or antagonistic (>4) (Humphries, 2016). This methodology is called Lowest FIC Index (Lowest Fractional Inhibitory Concentration Index) (Humphries, 2016).

3 Statistical analysis

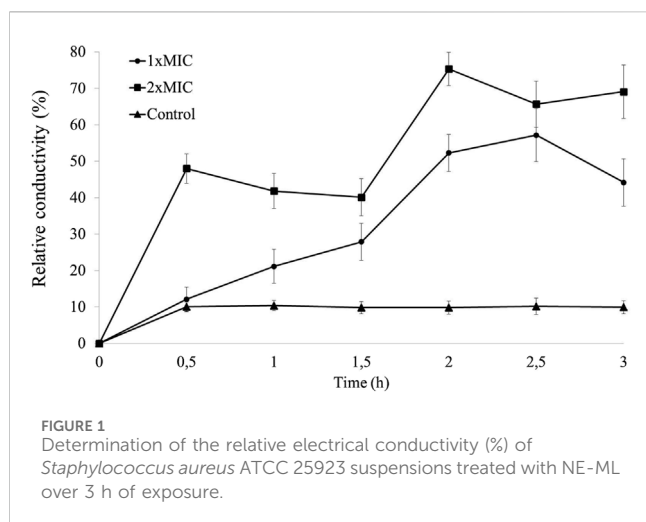
One-way analysis of variance (ANOVA) and Tukey tests were carried out to determine significant differences ($p < 0.05$) between the means using Microsoft EXCEL.

4 Results and discussion

4.1 Cell membrane permeability

An alteration in the electrical conductivity of the bacterial solution treated with 1xMIC and 2xMIC was detected (Figure 1).

The 1xMIC of NE-ML against *S. aureus* ATCC 25923 was confirmed to be 5% (v/v), equivalent to 125 $\mu\text{g/mL}$ of encapsulated macela extract, as described by Machado et al. (2020). Therefore, the MIC of 2xMIC was 10% (v/v), equivalent to 250 $\mu\text{g/mL}$ of encapsulated macela extract.



While the control group (isotonic *S. aureus* without NE-ML treated with boiling water) maintained stable relative conductivity over time (between 9.8% and 10.4%), the NE-ML-treated groups showed a considerable increase in relative electrical conductivity in the initial minutes of exposure. The 1xMIC group exhibited a gradual increase in relative electrical conductivity, peaking at 2.5 h of exposure (57.19%). The 2xMIC group showed a steep initial increase at 0.5 h (48.02%) and higher relative conductivity at 2 h of exposure (75.34%). These results suggest that the permeability of the *S. aureus* cell membrane was altered after treatment with NE-ML, causing the leakage of intracellular electrolytes into the culture medium, as expressed by an increase in the conductivity values of the bacterial suspensions.

The change in membrane permeability observed seems to be dependent on the concentration of the nanoformulation because we found that an increase in the concentration of NE-ML was correlated with an increase in relative electrical conductivity from 12.18% to 48.02%, an increase of about four times. Similar results were found by Wei et al. (2023) when evaluating the activity of madecassic acid isolated from *Centella asiatica* on the cell membrane of *S. aureus*. This increase in relative electrical conductivity over time is considered an indicator of changes in the permeability of the plasma membrane. Relatively small changes can negatively affect metabolism and lead to cell death. If plasma membrane permeability is affected, intracellular electrolytes will leak into the culture medium, and the conductivity of the supernatant will reflect it (Wei et al., 2023).

The viscosity of the plasma membrane directly influences its associated functions, including passive permeability of hydrophobic molecules, active solute transport, and protein-protein interactions (Zhang and Rock, 2008). Therefore, it is essential that the membrane remain intact for the performance of these functions without causing metabolic dysfunction (Hartmann et al., 2010) and subsequent loss of cellular functionality (Pérez-Peinado et al., 2018).

The plasma membrane delimits the cytoplasm and controls the diffusion of small molecules and secreted proteins between intracellular and extracellular spaces (Strahl and Errington, 2017), acting as a regulatory barrier to the passage of small intracellular ions that influence bacterial membrane potential. Intracellular potassium contributes to the maintenance of cell turgor and membrane potential, as well as pH homeostasis, in addition to

influencing enzymatic activity (Stautz et al., 2021). An increase in conductivity indicates an increase in bacterial membrane permeability, leading to the leakage of intracellular constituents, such as the electrolytes K^+ , Ca_2^{+} and Na^+ (Li and Yu, 2015).

4.2 Cell membrane integrity

Mean values of optical densities were obtained from screening for the detection of absorbing material which at 260 and 280 nm is indicative of the loss of macromolecules, such as nucleic acids (Lucena-Aguilar et al., 2016) and proteins (Stoscheck, 1990), resulting from the alteration of plasma membrane integrity of *S. aureus* ATCC 25923 treated for 2 and 4 h with NE-ML (Table 2).

Low optical density values at 260 nm and 280 nm were found in the control group, suggesting that the bacterial cell was intact in the culture conditions. However, in the groups treated with NE-ML (1xMIC and 2xMIC), a significant increase in the optical density of the bacterial suspensions was observed. By increasing the concentration of NE-ML from 1xMIC to 2xMIC, higher optical density values occurred in bacterial suspensions, in turn indicating a greater release of macromolecules with the increase in product concentration. Following the detection of absorbent material at 260 and 280 nm, total protein was performed in the bacterial suspension.

In this assay, the bacterial suspension treated with 1xMIC resulted in an increase of total protein content from 97.15 $\mu\text{g}/\text{mL}$ at 2 h of exposure to 138.63 $\mu\text{g}/\text{mL}$ after 4 h (Figure 2). In the group treated with 2xMIC, the content increased from 196.41 $\mu\text{g}/\text{mL}$ to 231.96 $\mu\text{g}/\text{mL}$ over the same period of exposure to NE-ML. Compared to the control group, 1xMIC and 2xMIC groups at both time points (2 and 4 h) showed a significant difference in total protein concentration ($\mu\text{g}/\text{mL}$) ($p < 0.05$). These results indicate damage to the plasma membrane integrity of the *S. aureus* ATCC 25923 in proportion to the concentration of NE-ML in the culture medium.

Similar studies that evaluated the antimicrobial activity of cinnamon essential oil against *S. aureus* also detected the release of total proteins in the culture medium. The authors observed that the protein concentration increased over time and in response to the increase in cinnamon essential oil concentration. The presence of these cellular constituents in the culture medium possibly indicates damage to and alteration in plasma membrane integrity (Zhang et al., 2016).

Some bioactive compounds present in the macela extract of the nanoemulsion under study, e.g., the flavonoids quercetin and luteolin, have been associated with structural damage responsible for the loss of bacterial membrane function. Similarly, the flavonoids epicatechin, quercetin and its glycosides extracted from white guava leaves also exhibited bacteriostatic activity against *S. aureus* through mechanisms that disrupt the structure and function of the bacterial cell membrane (Zhang et al., 2020).

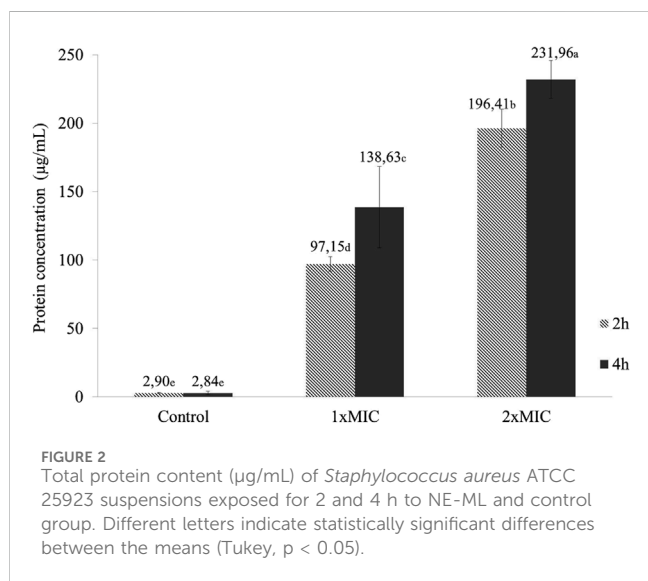
4.3 Effect of macela-nanoemulsion on the ultrastructures of *Staphylococcus aureus*

Transmission electron micrographs revealed alterations in the ultrastructure of *S. aureus* ATCC 25923 owing to the action of NE-

TABLE 2 Detection of absorbing material at 260 and 280 nm in suspensions of *Staphylococcus aureus* ATCC 25923 treated with NE-ML for 2 and 4 h.

Release of cellular constituents				
Treatments	OD260 nm		OD280 nm	
	2 h	4 h	2 h	4 h
Control	0.039 ± 0.001 _c	0.037 ± 0.001 _c	0.024 ± 0.002 _c	0.022 ± 0.001 _c
1xMIC	0.238 ± 0.044 _b	0.247 ± 0.077 _b	0.240 ± 0.035 _b	0.244 ± 0.068 _b
2xMIC	0.477 ± 0.061 _a	0.540 ± 0.101 _a	0.498 ± 0.056 _a	0.552 ± 0.085 _a

Different letters within the same column indicate statistically significant differences between the means (Tukey, p < 0.05).

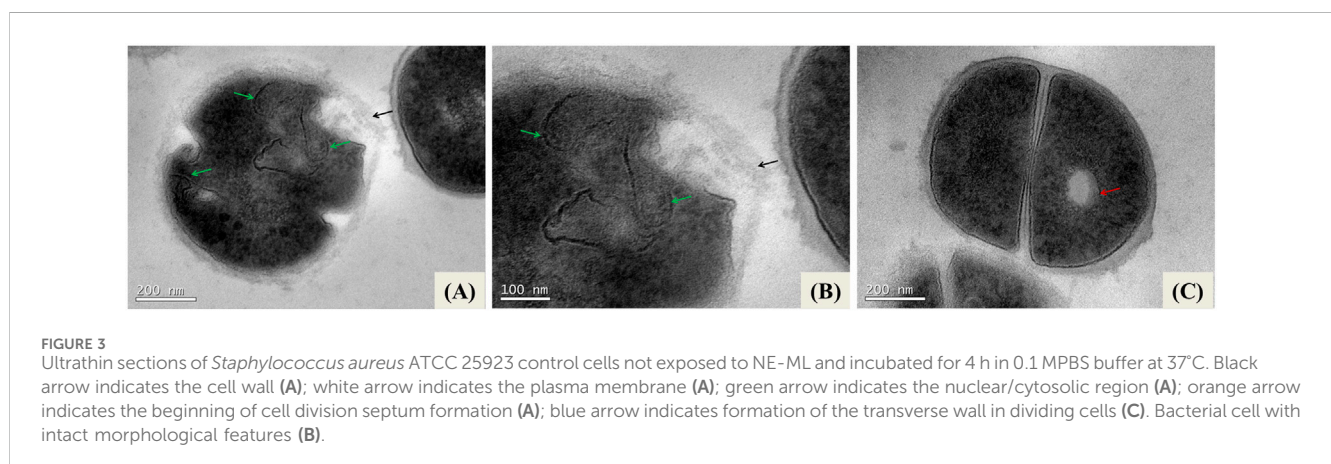


ML. Bacteria from the control group not exposed to NE-ML (Figure 3) exhibit uniform characteristics with intact and well-defined cell walls and plasma membranes. The relief of the cell wall showed a rough to smooth characteristic. The plasma membrane appeared as an electron-dense layer, just below the cell wall. It was also possible to observe the cytosol containing bacterial genetic material clustered in the central region of the cells with electron-dense and electron-transparent areas. Cells

undergoing division displayed standard septation characteristics in different phases with the presence of initial septa up to the formation of the complete transverse wall. These ultrastructure characteristics are consistent with the description of control *S. aureus* in other investigations (Santhana Raj et al., 2007; Grigor'eva et al., 2020).

However, bacteria exposed to NE-ML concentrations for 2 h in 0.1 M PBS buffer at 37°C show structural alterations which are intensified with the increase in NE-ML concentration from 1xMIC to 2xMIC (125 and 250 µg/mL of nanoencapsulated macela extract, equivalent to 5% and 10% of NE-ML v/v, respectively). From ultrathin sections of *S. aureus* ATCC 25923 exposed to 1xMIC of NE-ML for 2 h (Figure 4), we observed changes in the uniformity of cytosolic density with areas of electron-dense and electron-transparent areas. Cells exhibit expression of cell wall regions, and many areas of the plasma membrane lose their conformity. It is also possible to observe well-defined and pronounced electron-transparent regions (hereinafter referred to as electron-transparent vacuoles).

When increasing exposure of the bacteria to 2xMIC of NE-ML for 2 h (Figure 4), it is possible to observe a greater presence of electro-transparent vacuoles in the bacterial cells. Changes in the plasma membrane intensify and show invaginations towards the intracellular region. It is also possible to observe alterations in the bacterial cell division pattern. The cell wall continues to thicken, and the cell membrane loses structure and definition, denoting significant cell deformation compatible with lysis.



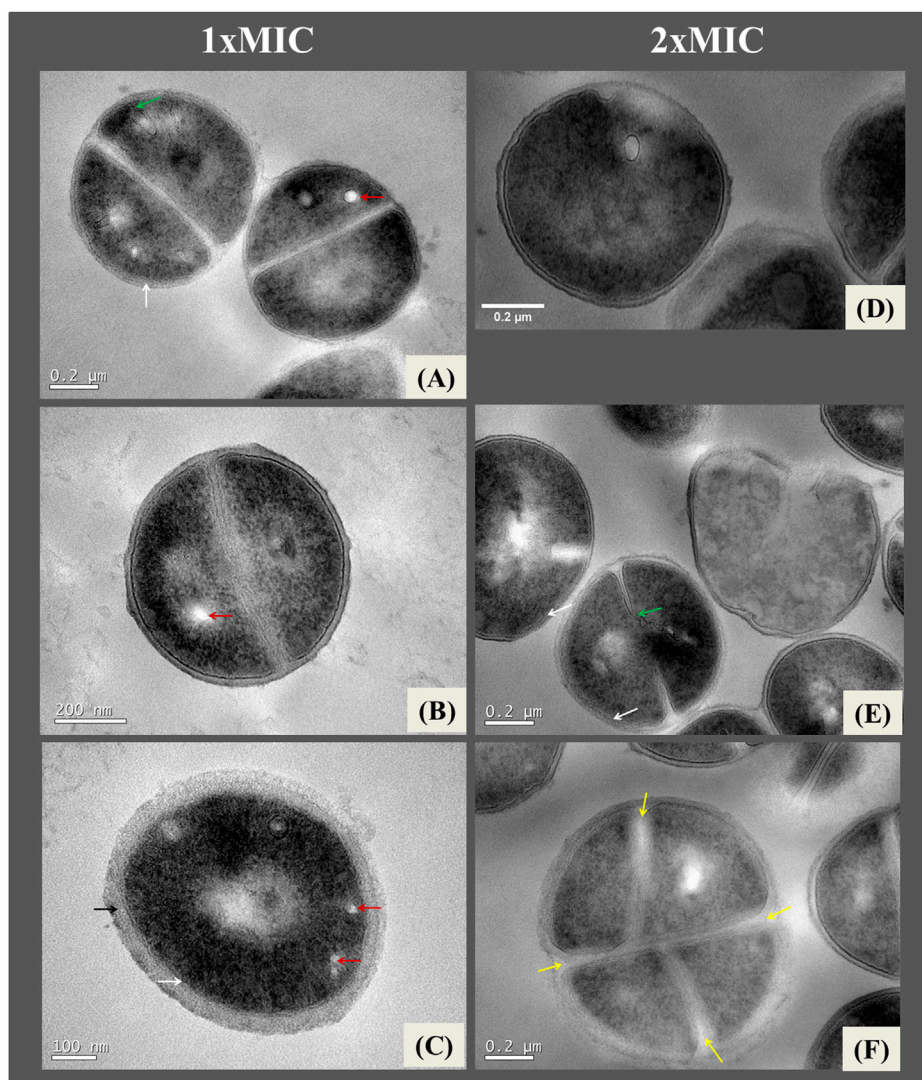


FIGURE 4

Ultrathin sections of *Staphylococcus aureus* ATCC 25923 exposed to 1xMIC (A–C) and 2xMIC (D–F) of NE-ML and incubated for 2 h in 0.1 M PBS buffer at 37°C. Black arrows indicate thickening of the cell wall; white arrows indicate loss of definition of the plasma membrane; red arrows indicate electron-transparent regions (vacuoles); green arrows indicate invaginations of the plasma membrane into the bacterial intracellular region; yellow arrows indicate changes in the pattern of cell division.

Figure 5 illustrate changes in bacteria exposed to the nanoformulation for 4 h at concentrations of 1xMIC and 2xMIC of NE-ML in 0.1 M PBS buffer solution at 37°C. In cells treated with 1xMIC of NE-ML after 4 h (Figures 5A–C), invaginations of the plasma membrane and some areas of the cell wall with loss of structure and definition were found (Figures 5A, B). The cells began to show a very pronounced electron-transparent area in the central region of the cytosol (Figure 5C). Increasing the concentration of NE-ML to 2xMIC for 4 h (Figures 5D–I) led to an increase in cell density, along with darkening of the cytosol region. The cells continued to show invaginations of the cell membrane (Figures 5D, E). The cells began to show damage and disorganization in the cytoplasm with several amorphous and electron-dense clusters within the entire intracellular structure much more pronounced when compared to cytoplasmic damage and disorganization of bacteria exposed for 2 h (Figure 5I).

We can also observe that the electron-transparent vacuole in the central region of the cell appears more pronounced after 4 h of exposure to NE-ML (Figure 5F). Some areas of the plasma membrane lose definition. In the group treated with 2xMIC of NE-ML, more pronounced changes and disorganization in the cell division pattern were also noticeable. Some daughter cells lose their structure, and the cell division septa are misaligned and have low-density material (Figures 5G, H), indicating that the areas of septal formation are affected by NE-ML.

Micrographs of *S. aureus* exposed to the nanoformulation revealed significant structural alterations which became more pronounced with increasing concentration and exposure time, as indicated by the thickening of the cell wall and pronounced deformities in regions of the plasma membrane. Results of the release of cellular constituents and electrical conductivity of bacterial solutions exposed to different concentrations of NE-ML

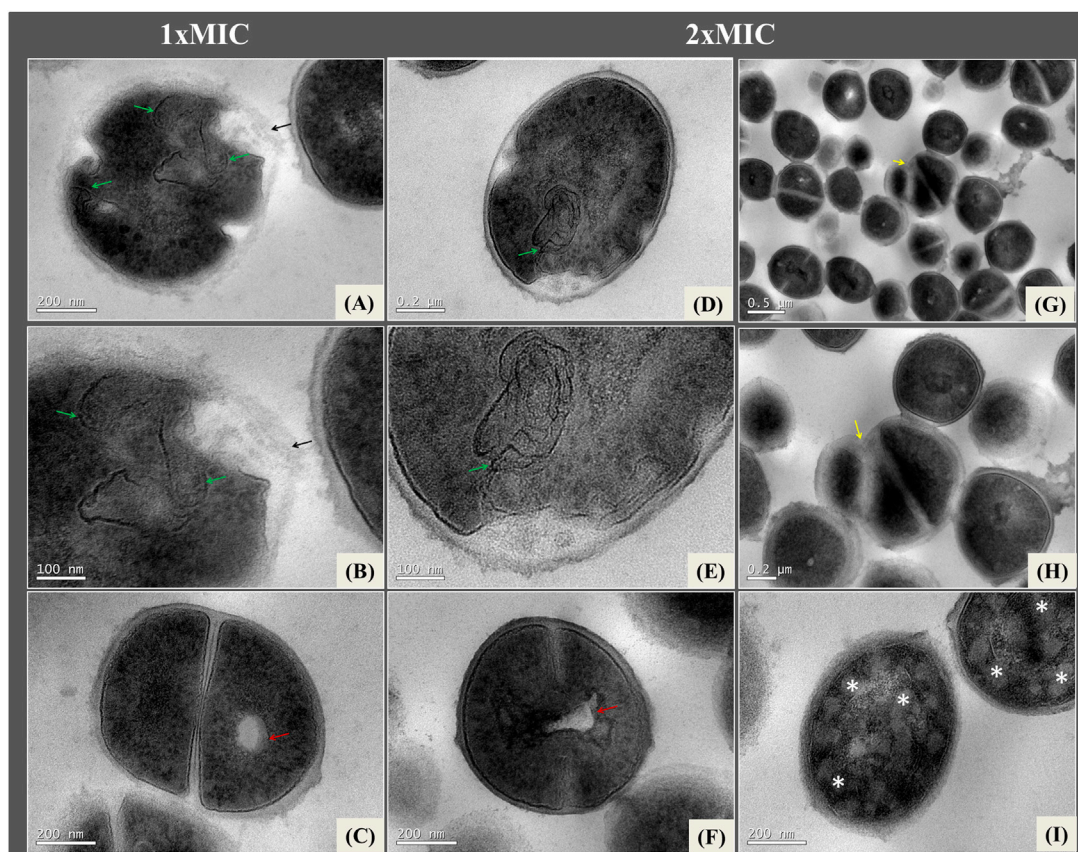


FIGURE 5
Ultrathin sections of *Staphylococcus aureus* ATCC 25923 exposed to 1xMIC (A–C) and 2xMIC (D–I) of NE-ML and incubated for 4 h in 0.1 M PBS buffer at a temperature of 37°C. Red arrows indicate electron-transparent vacuoles in the cytoplasm; green arrows indicate plasma membrane invaginations; black arrows indicate loss of cell wall definition; yellow arrows indicate changes in cell division pattern that are misaligned with electron-transparent material; * indicate cells with disorganization in the intracellular region and electron-transparent areas in the cytoplasm.

may be recalled. When combined, these findings suggest that the mechanism of action of the macela nanoformulation involves altering the integrity of the plasma membrane and increasing its permeability. These structural changes are responsible for the leakage of electrolytes and intracellular constituents, compromising essential bacterial components.

Other investigations have shown that such products as antimicrobial peptides like gramicidin S (GS) and gramicidin S (GS) and PGLa (peptidyl-glycylleucine-carboxyamide) interact with the bacterial membranes of *S. aureus*, increasing their permeability and destabilizing them with a decrease in the ability of bacteria to regulate turgor pressure in low ionic strength media (Hartmann et al., 2010). The formation of mesomeric structures surrounded by intracellular membranes was also observed, suggesting that this alteration occurs as an adaptation to the increased cell membrane area after binding to the antimicrobial peptide. These effects are similar to those observed in bacteria treated with gentamicin antibiotics which can bind to ribosomes and produce defective proteins, leading to bacterial death. Gentamicin affects the function of the cell membrane, resulting in the leakage of sodium, potassium, amino acids, and other cellular constituents (Anandabaskar, 2021).

Exposure to NE-ML may have also disrupted crucial cellular processes, such as DNA replication, chromosomal segregation, and enzymatic activity involved in peptidoglycan synthesis, all of which are essential for bacterial cell formation and division. It is not yet fully understood how the effects of many antibiotics can inhibit molecular processes that result in cell death. A growing body of evidence suggests that bacterial death occurs through highly complex and regulated mechanisms, very similar to the apoptosis mechanism in eukaryotic cells (Bayles, 2007). Some studies indicate that autolysis of *S. aureus* may occur in biofilm environments, possibly regulated by the *cidABC* and *lrgAB* operons, but the mechanisms are still poorly understood (Sadykov and Bayles, 2012).

We cannot assert that exposing bacteria to NE-ML directly stimulates the bacterial autolysis process. However, this hypothesis requires further investigation, especially through chromosomal analyses that assess the impact of NE-ML on these structures. Therefore, it is possible to indicate potential damage caused by NE-ML. This is relevant because, in a previous assessment of NE-ML against *S. aureus* MRSA biofilms *in vitro* (Machado et al., 2020), it was possible to reduce over 64% of the cellular mass, disrupting the integrity of the biofilm structure. Additionally, NE-ML demonstrated the ability to remove previously formed biofilm over 48 h.

We also observed that increasing the concentration of NE-ML extended cell damage to the cytosol which was marked by density alteration and the presence of electron-dense vacuoles, as well as altered bacterial division process, as observed by the presence of disorganized daughter cells and altered septation patterns. For bacteria to proliferate and ensure cell division, they need to go through consecutive cycles of growth and division, coordinating DNA replication, segregation of new chromosomes, and synthesis of new cell walls until completing cell division. In *S. aureus*, DNA replication, and peptidoglycan synthesis involve proteins and protein complexes that play a crucial role in regulating these processes (Barbuti et al., 2023). The initial phases of bacterial cell growth and division are marked by septation, a process that involves a coordinated invagination of the cytoplasmic membrane, peptidoglycan, and cell wall into the cytoplasm in the centripetal region of the bacterial cell (Paul et al., 1995; Touhami et al., 2004). In normal bacterial cells, the cell wall with the peptidoglycan of the mother cell remains external to the cell, while a new peptidoglycan wall grows in the septal region to the daughter cells. The lipid membrane defines the edges of the septal plate, while the peptidoglycan material of daughter cells forms the external cell wall, merging with the peptidoglycan of the mother cell's external wall during septum formation (Erickson, 2017).

4.4 Modulation of the antimicrobial activity of beta-lactam antibiotics and aminoglycosides in association with macelanoemulsion

According to the MIC breakpoint ($\mu\text{g/mL}$) (CLSI, 2019), we obtained bacterial profiles sensitive to oxacillin, resistant to penicillin, and sensitive to gentamicin. The sensitive category refers to an infection that can be treated with the recommended antibiotic dose for the type of infection and infecting species, while strains considered resistant are not inhibited by achievable systemic concentrations of antimicrobials in normal therapeutic regimens (CLSI, 2018). Penicillin-resistant bacterial strains were selected to investigate the modulatory capacity of antibiotics in combination therapy with NE-ML as a potential way to reverse bacterial resistance. The individual and combined MIC results of the tested antimicrobials, which are needed to calculate the ΣFIC index, are presented in Table 3.

Analysis of these data allowed us to determine whether NE-ML combined with oxacillin, penicillin, and gentamicin against *S. aureus* resulted in synergy, indifference, or antagonism (Table 4).

Most of the combinations tested between NE-ML and the different antibiotics resulted in interactions categorized as indifferent ($\Sigma\text{FIC} >0.5$ and ≤ 4). These interactions are so named because the effect of the tested combination is equal to the effect of the most active antimicrobial (EUCAST, 2000). The wild-type *S. aureus* strains studied (O1, P1, P2, G1, and G2) may exhibit genomic diversity, responding differently to antimicrobial action. Although synergistic interactions are most desired in the clinical context for combined therapy application (Yang et al., 2017), with a significant reduction in used concentrations, indifferent interactions can be valuable for preserving the effectiveness of existing antibiotics. Some antibiotics may present undesirable toxic effects, but when included

in a combined therapy system, the degree of side effects could be reduced, while, at the same time, maintaining good antimicrobial activity (Wang et al., 2022). Although the mechanisms of resistance to combined therapy are complex at a population level (Sullivan et al., 2020), combined antimicrobial therapy can also be considered to minimize or block antimicrobial resistance mechanisms and broaden the spectrum of drug activity (Dhanda et al., 2023).

A synergistic interaction between NE-ML and gentamicin was found (G2, $\Sigma\text{FIC} 0.31 \pm 0.00$). In synergism, the effects of the combination exceed the sum of the effects of the individual compounds (EUCAST, 2000). Here, we observed a 16-fold decrease in the concentration of NE-ML, from 125 $\mu\text{g/mL}$ (MIC alone) to 7.8 $\mu\text{g/mL}$ (MIC in combination), and a 4-fold decrease in the concentration of gentamicin, from 1 $\mu\text{g/mL}$ (MIC alone) to 0.25 $\mu\text{g/mL}$ (MIC in combination). Synergy is less common than other antimicrobial interactions because it may be related to different mechanisms of action affecting different targets in bacterial cellular components. It can also be related to specific genes, gene sets, or regulatory elements (Sullivan et al., 2020).

Synergistic interactions between different antibiotics can occur when they target the same cellular process by inhibiting a metabolic pathway in two subsequent steps, either when antibiotics physically interact by binding to the same target, or when antibiotics target different cellular processes, and one modulates the cellular uptake of the other. This modulation of drug uptake and efflux by bacteria is directly or indirectly conditioned by another drug, acting at different sites, i.e., membrane pores, efflux pumps, membrane potential, proton motive force (PMF), the force that moves protons across membranes against their concentration gradient, from areas of higher concentration to areas of lower concentration, and outer membrane composition (Roemhild et al., 2022). The bioavailability modulation model emerged as a concept in the 1960s, and it holds that the activity of one antimicrobial allowed the increase in the concentration of the other (Sullivan et al., 2020). For example, it has been observed that colistin, when binding to lipid A, causes cracks in the membrane and induces increased intracellular accumulation of other antibiotics, including rifampicin (Brennan-Krohn et al., 2018).

Throughout this investigation, we gathered evidence that led us to identify that the mechanism of action of NE-ML is related to the bacterial plasma membrane and that damage subsequently extends to other cellular structures, i.e., cytosol, cell wall, as well as bacterial cell division. These events result in the disruption of bacterial homeostasis with significant alteration of permeability and cellular integrity, as observed through electrolyte and macromolecular constituent leakage. Thus, the synergistic activity between NE-ML and gentamicin, as characterized by the use of lower doses of both antimicrobials, can be explained by uptake modulation. The action of NE-ML on the plasma membrane allowed gentamicin to increase uptake into the bacterial cytoplasm by altering its integrity and permeability, resulting in its action on intracellular targets with a lower concentration. Aminoglycoside antimicrobials, like gentamicin, irreversibly bind to the 30s ribosomal unit, affect mRNA translation, and lead to the incorporation of incorrect amino acids into the polypeptide chain. This results in the production of defective proteins, which

TABLE 3 Individual and mixed MICs of the antimicrobials on *Staphylococcus aureus*.

<i>S. aureus</i>	Individual MIC ($\mu\text{g/mL}$)				Mixed MIC ($\mu\text{g/mL}$)			
	NE-ML	OXA	PEN	GEN	NE-ML	OXA ^a	PEN ^b	GEN ^c
ATCC 25923	125	0.25	—	—	62.5	0.25	—	—
O1	125	0.25	—	—	62.5	0.25	—	—
P1	125	—	0.5	—	31.25	—	0.5	—
P2	125	—	0.25	—	62.5	—	0.125	—
G1	125	—	—	0.5	62.5	—	—	0.125
G2	125	—	—	1	7.8	—	—	0.25

^aMIC, of OXA, combined with concentrations between 7.81 and 750 $\mu\text{g/mL}$ of NE-ML.

^bMIC, of PEN, combined with concentrations between 7.81 and 750 $\mu\text{g/mL}$ of NE-ML.

^cMIC, of GEN, combined with concentrations between 7.81 and 750 $\mu\text{g/mL}$ of NE-ML.

TABLE 4 ΣFIC index.

<i>S. aureus</i>	Antimicrobials tested	ΣFIC index ^a	Result
ATCC 25923	NE-ML + OXA	1.5 \pm 0.00	Indifferent
O1	NE-ML + OXA	1.5 \pm 0.00	Indifferent
P1	NE-ML + PEN	1.25 \pm 0.00	Indifferent
P2	NE-ML + PEN	0.87 \pm 0.35	Indifferent
G1	NE-ML + GEN	0.75 \pm 0.00	Indifferent
G2	NE-ML + GEN	0.31 \pm 0.00	Synergistic

^aThe ΣFIC , index of each microplate was calculated, and the average of the replicates was used in the subsequent analyses (Bonapace et al., 2002) to define the interactions as synergistic (≤ 0.5), indifferent (>0.5 and ≤ 4), or antagonistic (>4) (Humphries, 2016). The interpretation of Interactions can be done through different methodologies with considerable variation between them. In the study, the interpretation method used was the lowest FIC, index among all non-turbid wells along the turbidity/non-turbidity interface (Humphries, 2016), which demonstrated the best synergy detection capacity (83%, $p < 0.0001$) compared to other methodologies for interpreting modulation tests with the checkerboard test (Bonapace et al., 2002).

play an important structural role essential for bacterial function and viability (Spinosa et al., 2017).

One of the main challenges in the search for new antimicrobials is related to the low cellular permeability of antibiotics (Murugaiyan et al., 2022). The development of drug administration or delivery systems that aim to improve this aspect, facilitating the entry of the antimicrobial into the bacterial intracellular space, proves to be a promising approach to overcoming this obstacle. Owing to their size, adaptability and stability in physiological fluids, nanostructured systems, e.g., metal-based nanoparticles, polymers, liposomes, or carbon-based, represent an option for transporting antimicrobials to hydrophilic and hydrophobic drug molecules (Pham et al., 2019). NE-ML was physically characterized as having an average particle size around 200 nm, monodisperse distribution (narrow size distribution) ($\text{PdI} < 0.2$), encapsulation efficiency of extract compounds in nanoparticles exceeding 94%, and physical stability over 160 days at room temperature (Machado et al., 2020). Furthermore, nanoparticles also exhibit the ability to inhibit biofilm formation (Mu et al., 2016), another major problem associated with controlling infections caused by *S. aureus*.

It is important to highlight a significant characteristic of nanoparticle application in the therapeutic treatment of subclinical bovine mastitis, often caused by intracellular infections of *S. aureus*. Some classes of antibiotics have limited

efficacy against intracellular pathogens because of low intracellular accumulation in eukaryotic cells, such as beta-lactams and aminoglycosides, or because of weak retention inside cells, like macrolides and fluoroquinolones (Abed et al., 2015). On the other hand, nanoparticles transporting antimicrobial molecules into the interior of eukaryotic cells can be taken up by phagocytic cells, resulting in a decrease in intracellular bacterial count (Abed et al., 2015). NE-ML has demonstrated the ability to inhibit biofilm formation by planktonic bacteria with a mass reduction of over 64% (biofilm MIC of 25% v/v), eradication capacity with a mass reduction of over 73% (minimum biofilm eradication concentration - MBEC, of 80% v/v), as well as the ability to remove previously formed biofilm over 48 h (Machado et al., 2020).

Nanostructured systems, such as the NE-ML, are considered effective nanocarriers, enhancing the stability, protection, and bioavailability of the nanoencapsulated compounds. They also increase penetration, solubility, dissolution, and permeation in the biomembrane (Harwansh et al., 2019). The antimicrobial activity of plant extracts, especially those rich in phenolic compounds like flavonoids and their derivatives, can occur through various mechanisms, including inhibition of function, porins inhibition, inhibition of nucleic acid synthesis, inhibition of energy metabolism, and inhibition of attachment and biofilm formation (Xie et al., 2015; Yuan et al., 2021).

The association of antimicrobial activity with changes in permeability and cell membrane rupture, leading to leakage of intracellular content (Álvarez-Martínez et al., 2021), has been explored and linked to the presence of hydroxyl groups at specific locations on aromatic rings (Xie et al., 2015). This characteristic can significantly alter membrane permeability and subsequent affinity with external and internal binding sites in bacteria.

Flavonoid compounds are the primary class of polyphenols by having over 8,000 described molecules and different biological activities. The major phytochemical compounds isolated from macela inflorescences (*A. saturoioides*) and incorporated into the nanoemulsion under study (NE-ML) are 3-O-methylquercetin ($187, 3 \pm 0.1 \mu\text{g/mL}^{-1}$), achyrobichalcone (ACB) ($155.4 \pm 11.6 \mu\text{g/mL}^{-1}$), quercetin ($76.3 \pm 0.1 \mu\text{g/mL}^{-1}$) and luteolin ($30.4 \pm 0.0 \mu\text{g/mL}^{-1}$) (Machado et al., 2020). The antimicrobial action mechanisms of these compounds are not fully elucidated, but it is known that phenolic compounds are hydrophobic in nature and are therefore considered to be active on the membrane (Álvarez-Martínez et al., 2021). Interaction with the lipid bilayer can modify the function of the plasma membrane, leading to the formation of small pores that alter its permeability and fluidity. This can result in the displacement of lipids and proteins, altering the electrical potential and polarity of the bacterial membrane. These changes may be responsible for significant damage, including membrane and cell wall rupture, disruption of DNA and RNA synthesis and function, and disturbance of normal cell communication, leading to inhibition of biofilm formation (Basavegowda and Baek, 2022).

5 Conclusion

Nanoformulations have emerged as a promising strategy for molecule delivery owing to their nanometric size and physical characteristics. The crude macela extract is a complex matrix, primarily composed of flavonoid compounds, such as 3-O-methylquercetin, quercetin, luteolin and achyrobichalcone, responsible for its antimicrobial activity. We confirmed that the formulation acts on the cellular plasma membrane, resulting in alterations in its permeability and integrity. Its adjacent effects include changes in cytosolic content and cell wall, as well as impact on the bacterial cell division process. Another important point to highlight was its synergism when combined with the antimicrobial gentamicin. Besides demonstrating itself to be an efficient delivery system for phytochemical molecules, this macela nanoformulation offers a promising perspective regarding its potential to enhance the absorption of other drugs within bacterial cells.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

RR: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing—original draft, Writing—review and editing. BR: Investigation, Methodology, Validation, Writing—review and editing. LH: Conceptualization, Writing—review and editing. SK: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Visualization, Writing—review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnano.2024.1466988/full#supplementary-material>

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