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RESEARCH ARTICLE

Microencapsulated *Syzygium aromaticum* essential oil enhances β-lactam efficacy against multidrug-resistant *Staphylococcus aureus*

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ABSTRACT

Background and Aim: Antimicrobial resistance (AMR) is a major One Health concern, especially in dairy production systems where multidrug-resistant (MDR) *Staphylococcus aureus* limits mastitis control. Essential oils (EOs), such as *Syzygium aromaticum* EO, possess strong antimicrobial properties, but their volatility restricts stability. Microencapsulation enhances EO stability and functional performance. This study aimed to evaluate the antimicrobial effect of microencapsulated *S. aromaticum* EO (OESAM) combined with β-lactam antibiotics against MDR *S. aureus*. Additionally, bioinformatic analysis was used to explore the potential role of whey protein isolate (WPI) used in the microcapsule wall material in modulating antimicrobial action.

Materials and Methods: OESAM was prepared via complex coacervation using WPI and gum arabic as wall materials. Ten MDR *S. aureus* isolates from bovine mastitis were tested for minimum inhibitory concentrations (MICs) of OESAM, oxacillin, and meropenem using broth microdilution. Synergy was evaluated using the Checkerboard method and interpreted through the fractional inhibitory concentration index (FICI). A protein–protein interaction network was generated in STRING database to investigate molecular pathways potentially affected by amino acids in WPI.

Results: Microencapsulation produced stable microcapsules with a yield of 39.11%. The addition of OESAM significantly reduced the MICs of oxacillin and meropenem ($p < 0.05$), resulting in antimicrobial dose reductions of up to 2.7-fold. Synergistic and additive effects predominated among the isolates. These outcomes were further supported by their FICI values, which confirmed potentiation between OESAM and β-lactams. Bioinformatic analysis identified three major functional clusters: tryptophan biosynthesis, siderophore biosynthesis, and phosphorelay signal transduction, suggesting that amino acids present in WPI may interfere with metabolic and regulatory pathways of *S. aureus*, enhancing antimicrobial effectiveness.

Conclusion: OESAM substantially enhances β-lactam efficacy against MDR *S. aureus*, offering a promising strategy for reducing antimicrobial doses and combating resistance in veterinary practice. Microencapsulation improves EO stability and enables controlled-release, thereby enhancing antimicrobial activity. Further in vivo validation, toxicity assessment, and formulation development are recommended to facilitate practical applications in mastitis control and broader AMR mitigation.

Keywords: antimicrobial resistance, *Staphylococcus aureus*, fractional inhibitory concentration index, mastitis, microencapsulation, One Health, Synergy, *Syzygium aromaticum*, whey protein isolate.

INTRODUCTION

Antibiotic resistance continues to rise globally and is now recognized as one of the principal drivers of increased morbidity, mortality, and hospital admissions. It is considered a major threat to public health [1]. Antimicrobial resistance (AMR) affects humans, animals, and ecosystems, reinforcing the need for integrated and sustainable control strategies. Within this context, the One Health approach has become essential for

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coordinating actions across sectors. The World Health Organization and national health authorities emphasize the implementation of surveillance and mitigation programs, particularly in agricultural production systems where antimicrobial use is widespread [2]. In dairy herds, resistant strains of *Staphylococcus aureus* pose significant challenges to the treatment and management of bovine mastitis. Although mastitis commonly manifests as a subclinical infection, severe presentations such as gangrenous mastitis with life-threatening consequences may also occur [3]. S. aureus is notorious for causing persistent and chronic infections with low cure rates, even following antibiotic therapy [3]. These strains exhibit an exceptional capacity to develop resistance to nearly all antimicrobial classes used in clinical practice, especially β-lactam antibiotics, which are routinely employed to treat infections caused by Gram-positive bacteria [4]. β-lactams disrupt cell wall synthesis by inhibiting key enzymes, including transpeptidases, transglycosylases, and carboxypeptidases, required for peptidoglycan production, a vital structural component of the bacterial cell wall [5].

Given the rapid emergence of resistance that outpaces the development of new antimicrobials, interest in natural therapeutic alternatives has increased substantially. Essential oils (EOs) have gained prominence due to their natural origin, environmental compatibility, and diverse biological properties [6]. *Syzygium aromaticum* (clove) EO is widely utilized in traditional medicine and the food industry owing to its rich profile of bioactive constituents, including eugenol, β-caryophyllene, α-humulene, and eugenol acetate, which exhibit antioxidant, antibacterial, anti-inflammatory, antihypertensive, and potential anticancer activities [7, 8]. EOs are liquid and volatile at room temperature [6]. Microencapsulation has therefore emerged as a valuable technology to enhance their chemical, oxidative, and thermal stability [9]. This approach prolongs shelf life, preserves biological activity, improves physicochemical characteristics, and enables controlled-release of active compounds [10]. The technique relies on separating a coating material from a liquid phase to encapsulate the core substance [11]. A variety of coating agents, including proteins and polysaccharides, can be employed in microcapsule production. Current evidence indicates that EO microencapsulation is an efficient and economically feasible strategy across multiple industrial sectors [6], particularly in dairy and livestock systems facing high levels of AMR [12].

Although EOs, particularly *S. aromaticum* EO, exhibit substantial antimicrobial potential, their practical application in veterinary medicine remains limited due to challenges related to volatility, instability, and inconsistent antimicrobial performance. Microencapsulation has emerged as a promising strategy to overcome these limitations, improving stability, protecting volatile compounds, and enabling controlled-release. However, despite the demonstrated benefits of EO microencapsulation, there is a lack of studies evaluating its combined use with conventional antimicrobials, especially β-lactam antibiotics, against multidrug-resistant (MDR) *S. aureus*, a major pathogen responsible for persistent bovine mastitis. Additionally, the existing literature provides limited insight into the molecular mechanisms by which microencapsulation materials, such as whey protein isolate (WPI), may enhance antimicrobial activity. The functional contribution of WPI amino acids to bacterial interaction, metabolic interference, or modulation of antibiotic performance remains largely unexplored. Therefore, there is a critical need for integrated *in vitro* and in silico investigations that clarify how microencapsulated compounds influence antimicrobial efficacy and resistance pathways.

The aim of this study was to comprehensively evaluate the antimicrobial potential of microencapsulated *S. aromaticum* EO (OESAM) in combination with β-lactam antibiotics against MDR *S. aureus* strains isolated from bovine mastitis. Specifically, the study sought to (i) determine the minimum inhibitory concentrations (MICs) of OESAM, oxacillin, and meropenem individually and in combination; (ii) assess modulatory, synergistic, and additive effects using the fractional inhibitory concentration index (FICI); and (iii) investigate the possible mechanistic contribution of WPI 90%, used as a wall material in the microencapsulation process, through bioinformatic analysis of protein interaction networks in *S. aureus*. By integrating laboratory assays with *in silico* functional pathway analysis, this study aimed to provide a deeper understanding of how microencapsulation can enhance antimicrobial performance and support the development of alternative therapeutic strategies to combat AMR in veterinary medicine.

MATERIALS AND METHODS

Ethical approval

All procedures involving the collection and use of bovine milk samples were conducted in accordance with national and institutional guidelines for animal research and biosafety. The isolates used in this study originated from milk samples collected as part of routine mastitis monitoring and diagnostic activities, without requiring direct manipulation, restraint, or intervention in animals. No experimental procedures were performed on live animals.

The study protocol, including the use of bacterial isolates and microencapsulation procedures, was reviewed and approved by the Ethics Committee on Animal Use (CEUA) of the Federal University of Minas Gerais (UFMG), Brazil, under approval number [90/2018].

Additionally, all activities related to sample collection and isolate preservation were registered in the National System for Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN), under registration number A8D3D14, in compliance with Brazilian regulations.

Study period and location

The study was conducted from January to December 2024 at the Animal Health Laboratory, linked to the Institute of Agricultural Sciences (ICA) of the Federal UFMG, located on the Montes Claros campus, in the state of Minas Gerais, where all experimental stages and laboratory analyses relevant to the research were carried out.

Bacterial strains and their identification

Source of isolates: We used isolates of the genus from the Bacteriotheque of the Animal Health Laboratory of the Institute of Agricultural Sciences of the UFMG, Montes Claros campus, MG. These isolates were obtained from milk samples collected from teats positive for bovine mastitis from different herds in the northern region of Minas Gerais, between 2017 and 2023, within the scope of extension and scientific initiation projects duly registered in the National System for Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN), under registration number A8D3D14.

Reactivation and culture identification: Ten isolates of the *S. aureus* species were selected. The previously frozen isolates were reactivated in Brain Heart Infusion (BHI) Broth (Himedia, Thane, Maharashtra, India) and then plated on Tryptone Soya Broth (Himedia) to identify the strains. We selected pure colonies with the characteristic morphology of Gram-positive cocci grouped in clusters.

Confirmation by Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS): The fresh and pure cultures were subjected to MALDI-TOF mass spectrometry, using the Microflex™ MALDI-TOF MS equipment (Bruker Daltonics, Brazil), at the Aquaculture Laboratory of the Veterinary School of the UFMG, in Minas Gerais, Brazil [13].

AMR profile

Disk diffusion testing: To characterize multidrug resistance, the strains identified by MALDI-TOF as *S. aureus* were evaluated using the disk diffusion technique on Mueller-Hinton agar using the Sensibiodisc (São Paulo, Brazil) from the betalactam class, obtained from Laborclin: oxacillin (1 mcg), imipenem (10 mcg), meropenem (10 mcg), cefoxitin (30 mcg), and sulbactam + ampicillin (10 mcg) [14].

Criteria for multidrug resistance: Bacterial strains were considered MDR when they showed resistance to two or more antimicrobial agents.

Characterization of the EO of *S. aromaticum*

Source and storage: The Syzygium aromaticum Essential Oil (OESA) used in this study was previously purchased from Ferquima Indústria e Comércio Ltda®, lot 233, stored at controlled room temperature between 22°C and 25°C, and protected from light throughout the experiment, and characterized by chromatography.

Gas chromatography–mass spectrometry (GC–MS) Analysis: The chemical composition was analyzed at the Instrumental Chemistry Laboratory at ICA/UFMG - Montes Claros campus using a GC–MS system model 7890 coupled to a mass spectrometer (MS 5975C) (Agilent Technologies, USA) with a fused silica capillary DB5-MS column (30 m x 0.25 mm x 0.25 µm); helium (99.9999% purity) was used as the carrier gas at a flow rate of 1mL min-1. The injector was maintained at 220°C, at a flow rate split ratio of 1:5 and was then subjected to 60°C-240°C (3°C min-1) for 10 min. The system operates in full scan with an electron impact of 70 eV in the range of 45-550 (m/z). The compounds were identified by comparing mass spectra with data from the NIST 2.0 library (2009) and by relative retention indices, determined experimentally and compared with values reported in the literature [15, 16].

Major bioactive component: Eugenol was the main bioactive component present in the oil used (85%).

Microencapsulation procedure

Preparation of solutions and emulsion: OESA was used as the active material, and 1% gum arabic (GA; Êxodo Científica, Sumaré, Brazil) and 3% WPI (Sooro Renner Nutrição, Marechal Cândido Rondon, Brazil) were used as wall materials. The solutions were prepared individually in 250-mL volumetric flasks and magnetically homogenized at 79 × *g* and 25°C for 60 min. Then, 12.5 g (5%) of EO was added dropwise to the WPI solution

under stirring with an ultraturrax (T 25 digital ULTRA-TURRAX®, IKA, Brazil) at 1,500 rpm for 8 min. Then, the GA solution was slowly added, resulting in a final emulsion (500 mL) containing WPI, GA, and EO. The pH of the emulsion was adjusted to 3.75 using 1% (v/v) citric acid (Neon, Suzano, Brazil) to favor electrostatic interactions between macromolecules.

Microscopy and freeze-drying: The formation and morphology of the microcapsules were evaluated by optical microscopy (Physis, Santo André, Brazil) before and after cooling to 10°C [17]. Then, the microcapsules were freeze-dried to produce solid microcapsules. Freeze-drying was conducted in a Christ Alpha 1-2 LD freeze (IKA, Campinas, Brazil), under a pressure of 6.1 mbar, a temperature of –14°C, and a drying time of 48 h.

Evaluation of encapsulation performance: Oxidative stability, yield and microencapsulation efficiency of surface oil, as well as the morphology of solid microcapsules, were evaluated according to the procedure described by Eratte *et al.* [18], with methodological adaptations that will be detailed later.

Characterization of the microencapsulation

Yield calculation: The freeze-dried microcapsules were weighed on an analytical balance to determine the yield of the process, using equation (I):

Yield of microcapsules (%) = Mass of lyophilized microcapsules (g) / Total mass of whey + gum arabic + oil (g) × 100 (I)

Encapsulation efficiency procedures: The encapsulation efficiency was determined by quantifying the surface EO and the total content present in the microcapsules. A 3 g sample of microcapsules was added to 30 mL of Hexane (Êxodo Científica, Sumaré, Brazil) and shaken at 225 rpm for 5 min. The mixture was then filtered through filter paper with a pore size of 5 µm and the solid residue was washed three times with 10 mL of hexane to completely extract the surface oil. The resulting filtrate was concentrated in a rotary evaporator at 60°C to remove the solvent and recover the oil. The sample was then kept under a hood exhaust for 4 h to eliminate residual hexane.

The total OESAM content was determined using the acid digestion method described by Eratte *et al*. [18]. Briefly, 3 g of microcapsules were added to 30 mL of 4 N HCl solution and stirred at 225 rpm for 15 min. Afterward, 15 mL of hexane was added to the mixture, which was stirred again for 18 h at 25°C, allowing the oil to be completely extracted. The resulting solution was centrifuged at 24,471 × *g* at 10°C for 30 min using a Sorvall ST 16R centrifuge (Thermo Scientific, Osterode am Harz, Germany). The organic phase (hexane), which contained the dissolved oil, was recovered and concentrated on a rotary evaporator at 60°C. Finally, the sample was kept under a hood exhaust for 4 h to eliminate residual hexane.

The entire procedure was performed in independent duplicates to ensure the reproducibility of the results. Equations for surface oil, TO, and microencapsulation efficiency.

The percentages of SO, total oil (TO), and ME were determined using equations (II), (III), and (IV), respectively:

- SO = Ws / Wm × 100% (II)
- TO = Wt / Wm × 100% (III)
- ME = (Wt – Ws) / Wt × 100% (IV)

Where Wt and Ws are the mass values (g) of total and surface oil of the microcapsules, respectively, and Wm is the mass (g) of the microcapsules.

Antibacterial assays

Determination of MIC of OESAM: The MIC of OESAM was determined according to the methodology described by the Clinical and Laboratory Standards Institute, 2015b [14]. Briefly, solutions at concentrations of 170, 150, 130, 110, and 90 µl/ml of OESAM were prepared and applied to microtiter plates. The bacterial strains were standardized at 10^8 CFU/mL using the McFarland 0.5 scale and applied to the wells of the microplates, which were incubated at 37°C for 24 h. The MIC was read using the change in color of 1% 2,3,5-triphenyl tetrazolium chloride (TTC), whose red coloration indicated bacterial growth. Samples that showed no turbidity or coloration were plated on Petri dishes containing Plate Count Agar (Oxoid, Basingstoke, Hampshire, England), to confirm the reduction of viable colonies. The standard strain of *Staphylococcus* American Type Culture Collection (ATCC) 25923, grown in Brain Heart Infusion broth, was used as a positive control, while sterile BHI broth, without bacterial inoculation, was used as a negative control.

Determination of MIC of oxacillin and meropenem: Oxacillin and meropenem antibiotics were evaluated for their MIC against MDR strains of *S. aureus*. The MIC was determined using the broth microdilution method in 96-well microtiter plates according to the Clinical and Laboratory Standards Institute (CLSI) protocol (2015b), with [14]. Breifly, increasing concentrations of the antimicrobials were tested (1, 2, 4, 6, 8, 10, 12, 20, 30, 40, 50, 60, 70, 80,

90, 100 and 200 µL/mL). Bacterial strains were standardized based on the McFarland 0.5 scale (approximately 10^8 CFU/mL) and 20 µL were inoculated into each well. The S. aureus ATCC 25923 strain was used as a positive control and sterile BHI as a negative control. All tests were performed in triplicate. After incubation at 37°C for 24 h, 1% TTC was added and the mixture was incubated for 2 h. The absence of a change in the TTC color (from yellow to red) indicated the inhibition of bacterial growth.

Checkerboard microdilution method for OESAM–antibiotic associations

Combination testing procedure: Modulatory activity was assessed using the checkerboard method, as described by Lahmar *et al.* [19]. Analysis was conducted between MDR S. aureus strains with double combinations of oxacillin and meropenem. The first antimicrobial agent in the combination was serially diluted along the ordinate (vertical axis) of the microplate to enhance the joint action between the EO and the antibiotic, while the second agent was serially diluted along the abscissa (horizontal axis). A total of 49 different combinations of antibiotic concentrations were tested in association with the EO.

The concentrations evaluated ranged from 90 to 190 µL/mL for OESAM, 40 to 60 µL/mL for oxacillin, and 10 to 40 µL/mL for meropenem. The concentrations were established from the MIC of each compound, with successive dilutions up to 1/16, using a bacterial inoculum standardized at 10^8 UFC/mL using the McFarland 0.5 scale. The plates were incubated at 37°C for 24 h, after which the new MIC for each combination was determined. S. aureus ATCC 25923 was cultured in BHI broth as a positive control and sterile BHI broth free of bacterial inoculation was used to ensure the absence of contamination and confirm the sterility of the culture medium. All tests were carried out in triplicate.

FICI Calculation and interpretation: The FICI was used to interpret the results according to the methodology proposed by Siqueira *et al.* [20]. The FICI was calculated by summing the minimum FICs of the tested compounds. The FIC of each substance was determined by the ratio between the MIC obtained in combination and the MIC of the substance tested alone, according to the following equations:

- FIC Drug = Combined MIC / Isolated MIC (V)
- FIC OESAM = Combined MIC / Isolated MIC (VI)

The FICIs were interpreted as follows: Synergy: FICI ≤ 0.5; Additivity: FICI > 0.5 to 1; Indifference: 1 to 4; Antagonism: FICI > 4.

Antibacterial tests were conducted in triplicate using two independent analyses, and the results were expressed as means ± standard deviation.

Bioinformatic Analysis

Construction of protein interaction network: A protein interaction network was developed to evaluate the potential to potentiate the antimicrobial effect of the EO through microencapsulation and to analyze the action of the microcapsules on S. aureus. The microcapsules are composed of the active material, the EO of S. aromaticum, and the wall material, which is GA and WPI 90%, registered with the Ministry of Agriculture, Livestock and Supply (MAPA) under No. 0102/1328 - NCM 3502.20.00 - SAP P100103 and P700103.

STRING analysis and functional enrichment: The amino acids present in the composition of WPI 90% (aspartic acid, glutamic acid, alanine, arginine, cysteine, phenylalanine, glycine, histidine, isoleucine, leucine, lysine, methionine, proline, serine, tyrosine, threonine, tryptophan, and valine) were entered into the STRING V. 12.0 platform [21], selecting the S. aureus species. The previously formed protein interaction network, with a confidence score of 0.4 and all selected interaction sources, was expanded to include no more than 10 interactors in the first layer and no more than 30 in the second layer. The network was divided into 3 groups using K-means clustering. The functional enrichment of the network was obtained using Kyoto Encyclopedia of Genes and Genomes (KEGG).

The network was analyzed based on the scientific literature and *in vitro* tests.

Statistical analysis

The obtained data were subjected to descriptive analysis, including frequency counts, means, and standard deviations. The data obtained in the *in vitro* tests had a normal distribution (Shapiro-Wilk) and were statistically analyzed using analysis of variance, followed by the Tukey test at a significance level of 5% ($p \leq 0.05$). Statistical analyses were performed using Microsoft Excel 2016 (Microsoft Corp., Washington, USA) for descriptive statistics and PAST v5.3 (PAleontological Statistics, Natural History Museum, University of Oslo).

RESULTS

Microcapsule morphology and physical characteristics

Efficient microspheres require attention to basic criteria, including understanding the general properties of microcapsules. As for the coating material, the morphological and dimensional characteristics of the microcapsules, as determined by optical microscopy (Zeiss microscope coupled to an image capture system), are shown in Figure 1. The morphology and size of the microcapsules varied, with occasional clumping observed. The stability of the microcapsules in terms of pH was in the 3.75 range.

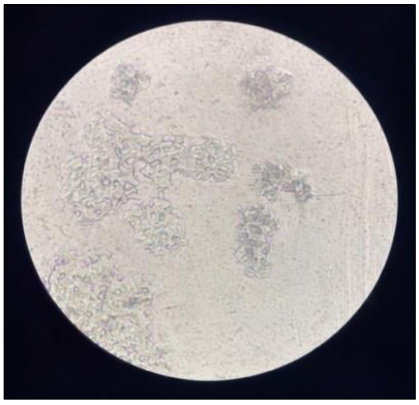

Figure 1: Optical micrographs showing the formation and morphology of microcapsules containing microencapsulated Syzygium aromaticum essential oil produced by complex coacervation using whey protein isolate and gum arabic as wall materials at pH 3.75. Images obtained under a 40× objective illustrate the overall size distribution, surface characteristics, and partial aggregation of the microcapsules, confirming successful microcapsule formation and structural stability prior to freeze-drying.

Microencapsulation yield and process performance

Regarding the performance of the microencapsulation process, a yield of 39.11% was obtained. This value is comparable to that reported by Carvalho *et al.* [17], who obtained a yield of 45.03% using the same complex coacervation methodology and the same active compound. This similarity suggests that the technique employed has satisfactory reproducibility.

Inhibitory activity of OESAM with β-lactam antibiotics

Figures 2 and 3 show the inhibitory activity of OESAM against oxacillin- and meropenem-resistant strains, respectively. Figure 2 shows a significant difference in the MICs of oxacillin alone and in combination with OESAM ($p < 0.05$). These results are encouraging in reducing the antibiotic dose to achieve the desired effect, which could contribute to reduced antimicrobial resistance in conventionally used antimicrobials for the treatment and control of bovine mastitis.

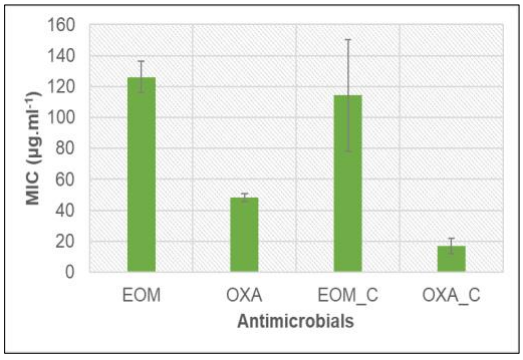

Figure 2: Minimum inhibitory concentrations (MICs) of microencapsulated Syzygium aromaticum essential oil (OESAM) and oxacillin tested individually and in combination against multidrug-resistant *Staphylococcus aureus* isolates. The combined treatment demonstrates a significant reduction in oxacillin MIC compared with oxacillin alone, indicating a modulatory and potentiating effect of OESAM on β-lactam activity ($p < 0.05$).

Notably, the MIC of meropenem was significantly reduced in the presence of OESAM ($p < 0.05$), as shown in Figure 3.

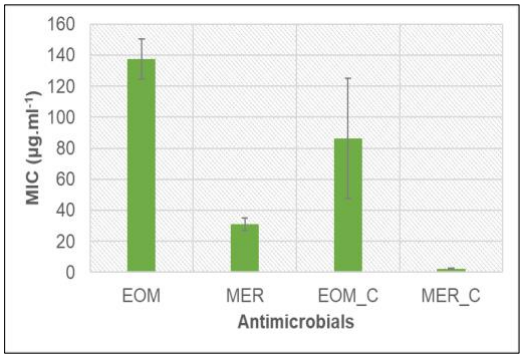

Figure 3: Minimum inhibitory concentrations (MICs) of microencapsulated Syzygium aromaticum essential oil (OESAM) and meropenem evaluated individually and in combination against multidrug-resistant *Staphylococcus aureus* isolates. The association with OESAM resulted in a significant reduction in the meropenem MIC compared with meropenem alone, demonstrating a clear potentiating effect of the microencapsulated essential oil on β-lactam antimicrobial activity ($p < 0.05$).

Modulatory effects and reduction in antimicrobial dose

The results are promising, as this is one of the first studies to demonstrate mean reductions of 12 and 51 µl/mL in the MICs of OESAM when combined with oxacillin and meropenem, respectively. Surprisingly, the average reductions in the MICs of oxacillin and meropenem when combined with OESAM were 31 and 29 µl/mL, respectively (Figure 4A). In this sense, there was an average reduction in antimicrobial doses of up to 2.7 times (Figure 4B) when combined with OESAM, constituting one of the first reports to demonstrate this modulatory capacity.

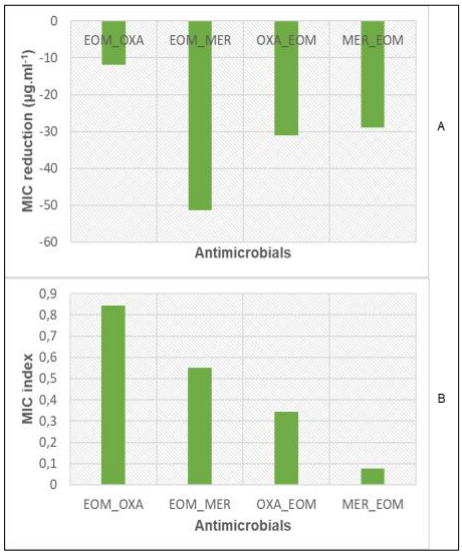

Figure 4: Effect of combining microencapsulated Syzygium aromaticum essential oil (OESAM) with β-lactam antibiotics against multidrug-resistant Staphylococcus aureus. (A) Reduction in the minimum inhibitory concentrations (MICs) of oxacillin and meropenem when tested in combination with OESAM compared with antibiotics alone. (B) MIC reduction index, calculated as the ratio of the MIC of the combination to the MIC of the antibiotic tested individually, illustrating the extent of antimicrobial dose reduction achieved through the combined treatment.

Synergistic and additive effects based on FICI analysis

Figures 5A and 6A show the fractional inhibitory concentration indices that determine the types of effects between OESAM and the antibiotics for oxacillin and meropenem, respectively. Although in some S. aureus strains an indifferent effect was observed between OESAM and antibiotics, synergistic and additive effects were observed (Figures 5B and 6B), indicating an important joint action between natural and chemical antimicrobials.

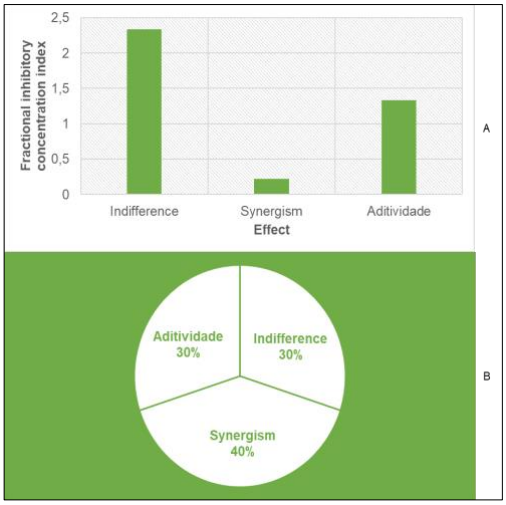

Figure 5: Interaction effects between microencapsulated Syzygium aromaticum essential oil (OESAM) and oxacillin against multidrug-resistant Staphylococcus aureus isolates based on the fractional inhibitory concentration index. (A) Distribution of FICI values obtained for each isolate, indicating the type of interaction. (B) Number of isolates exhibiting synergistic, additive, indifferent, or antagonistic effects, highlighting the predominance of synergistic and additive interactions between OESAM and oxacillin.

Protein interaction network analysis

Figure 7 shows the protein interaction network formed from the amino acids present in 90% WPI in S. aureus. The network is organized into 3 distinct clusters (represented by red, blue, and green). This is important because it reveals the molecular, biological, and functional mechanisms involved in these clusters.

Functional clusters and associated biological pathways

Table 1 shows the mechanisms involved in each cluster of the protein interaction network. Cluster 1 (red) represents the tryptophan biosynthesis pathway. Cluster 2 (green) is characterized by the siderophore biosynthesis pathway. Cluster 3 is defined by the phosphorelay signal transduction (PST) system.

Proteins were grouped using K-means clustering based on interaction density within the network. Functional enrichment was determined using KEGG pathway analysis. Source: STRING v12.0.

In the functional enrichment analysis of the protein interaction network using the KEGG pathway, most proteins in the network are involved in amino acid biosynthesis, secondary metabolite biosynthesis, and metabolic pathways (Figure 8).

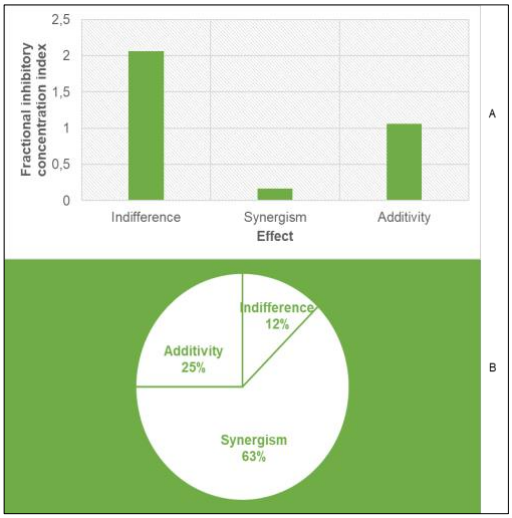

Figure 6: Interaction effects between microencapsulated Syzygium aromaticum essential oil (OESAM) and meropenem against multidrug-resistant Staphylococcus aureus isolates determined by the fractional inhibitory concentration index. (A) Distribution of FICI values for individual isolates, indicating the nature of the interaction. (B) Number of isolates exhibiting synergistic, additive, indifferent, or antagonistic effects, demonstrating the predominance of synergistic and additive interactions between OESAM and meropenem.

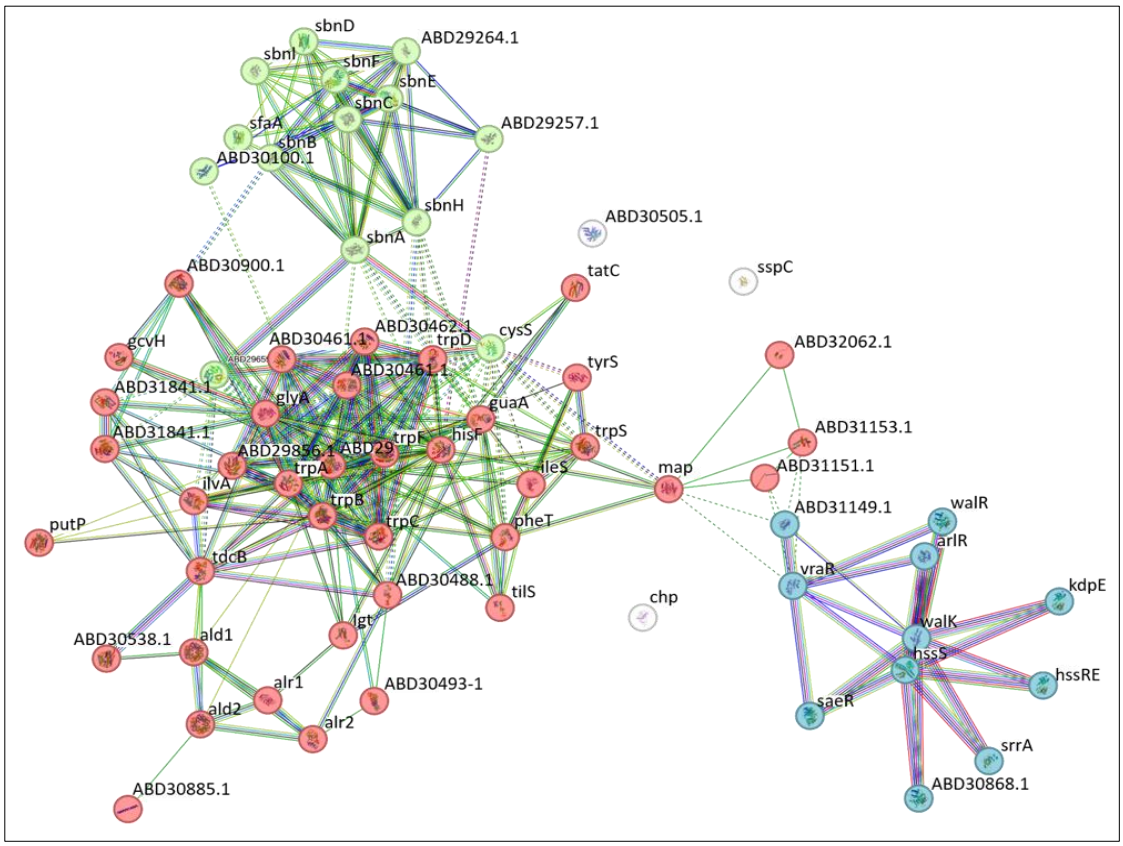

Figure 7: Protein–protein interaction network of *Staphylococcus aureus* generated using STRING v12.0 based on amino acids present in whey protein isolate (WPI 90%) used as the wall material for microencapsulation. The network is organized into three distinct clusters, representing pathways associated with tryptophan biosynthesis, siderophore biosynthesis, and phosphorelay signal transduction, highlighting potential molecular mechanisms through which WPI components may contribute to the potentiation of antimicrobial activity.

Table 1: Functional clustering of the Staphylococcus aureus protein–protein interaction network generated from amino acids present in whey protein isolate (WPI 90%) used as the wall material for microencapsulation, showing the main biological pathways and associated molecular functions identified by STRING v12.0 analysis.

Color	Cluster Id	Gene count	Description
	Cluster 1	28	Tryptophan biosynthesis
	Cluster 2	15	+ Siderophore biosynthesis
	Cluster 3	11	+ Phosphorelay signal transduction system

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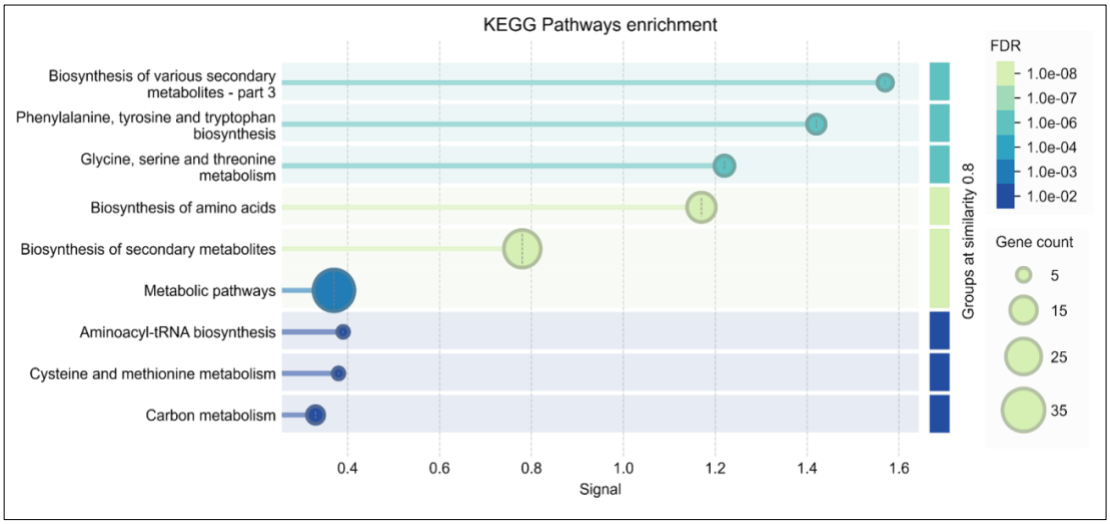

Figure 8: Functional enrichment analysis of the *Staphylococcus aureus* protein–protein interaction network derived from STRING v12.0. The enriched KEGG pathways indicate the involvement of amino acid biosynthesis, secondary metabolite biosynthesis, and central metabolic pathways, supporting the proposed role of whey protein isolate–derived amino acids in modulating bacterial metabolic and regulatory functions associated with antimicrobial potentiation.

Correlation between *in vitro* and *in silico* findings

Therefore, the results of the *in silico* analyses corroborate those of the *in vitro* analyses.

DISCUSSION

Characterization of microcapsules and structural stability

Analyses were carried out to characterize the microcapsules to assess the effectiveness and stability of the microencapsulation. The structural integrity of the microcapsules was maintained after the process, indicating they were sufficiently rigid, a crucial factor for protecting the OESA against external agents such as oxygen, light, and thermal variations. This characteristic significantly contributes to the physicochemical stability of the encapsulated compound [22].

Encapsulation efficiency and influencing factors

Efficiency is one of the most important quality parameters for microencapsulated essential oils. Some hypotheses can be proposed to explain the low efficiency of 40% in this study. The technological parameters of the freeze-drying stage can significantly influence process efficiency, requiring careful optimization. Xiao *et al.* [23] investigated the complex coacervation of lemon essential oil using different formulation conditions and technological variables, employing GA and type B gelatin as encapsulating agents. The encapsulation efficiency ranged from approximately 14% to 66%, as determined by UV-VIS spectrophotometry. They suggested that some of the observed losses could be associated with volatilization during the emulsification and coacervation stages [23]. Many studies have shown that emulsions with smaller droplet sizes result in microcapsules with greater microencapsulation efficiency by increasing the viscosity, which favors the rapid formation of the crust around the particle during drying [24].

The addition of gelatin, in addition to GA and sodium caseinate, promoted a significant increase in the retention of volatile compounds, as reflected by the increase in encapsulation efficiency from 28.6% to 65.9% in the study by Zhang *et al.* [25]. These results imply that the technological parameters and composition of the encapsulating matrixes have a critical influence on the EE process by complex coacervation, especially when associated with freeze-drying, and reinforce the importance of choosing the right biopolymers to stabilize emulsions and reduce volatilization losses. These findings suggest that optimizing technological and formulation variables improves not only process efficiency but also the stability and functional performance of microcapsules, which is essential for food, pharmaceutical, and veterinary applications.

Advantages of microencapsulation and antimicrobial potential

Considering the loss of bioactive compounds present in EOs, the microencapsulation process emerges as an effective strategy to protect them against volatilization. Due to its micrometric size, oxidative degradation, and environmental variations, it allows the controlled and targeted release of active ingredients, preserving their functional activity while intensifying β-lactam activity, demonstrating synergistic and innovative potential in

antimicrobial action, as shown in the current study [26]. Koc *et al.* [27] demonstrated that both free clove EO and microencapsulated clove EO inhibited the growth of Gram-positive bacteria, except Bacillus cereus, at the same concentration [27]. Cruz-Valenzuela *et al.* [28] reported that the microencapsulated EO demonstrated antibacterial activity against *Escherichia coli*, *Salmonella enterica* subsp. enterica serovar *Choleraesuis*, *Listeria monocytogenes*, and *S. aureus*, showing a MIC of 5.5 mg/mL for all the strains tested. To assess the effect of the oil on bacterial growth parameters, a sub-inhibitory concentration corresponding to half the MIC (2.75 mg/mL) was used [28]. Another study involving microparticles of eugenol, the main component of clove, resulted in significant antimicrobial activity against *S. aureus*, *Salmonella cholera suis*, and *E. coli*, in addition to preserving high antioxidant capacity, indicating that microencapsulation preserves and enhances the bioactive properties of the EO, configuring itself as a promising strategy for natural antimicrobial applications [29].

Microencapsulated lemongrass (*Cymbopogon citratus*) EO was studied by Assis *et al.* [30] and demonstrated significant antibacterial activity against *E. coli* (ATCC 8739), *S. aureus* (ATCC 6538), and *Salmonella enterica* (ATCC 6017) at a concentration of 80 µL/mL, in addition to maintaining stability when incorporated into broiler diets. These results reinforce the potential of OESAM as an effective strategy for application in animal production systems, combining stability and consistent antimicrobial effect.

Influence of surfactants and solvents on antimicrobial activity

In addition to the intrinsic activity of EOs, the influence of surfactants, such as Tween 20, Tween 80, and Triton X-100, and solvents, such as ethanol, present in the test media should be considered. These emulsifiers play an important role in facilitating the penetration of antimicrobial compounds into the bacterial cell wall and membrane structures. In this way, both surfactants and solvents can interfere with the efficacy of EOs or the response of microorganisms, thereby influencing the results of sensitivity tests [31]. Gram-positive bacteria, such as *S. aureus*, have a thicker layer of peptide glycans, giving the cell greater rigidity and protection. However, this layer may facilitate interaction with the coating layers of the microcapsules. In this way, the S. aureus bacteria could be eliminated more efficiently by releasing the oil directly closer to its wall, which facilitates the GA and WPI 90% particles' interaction/approach. This would increase the local concentration of the oil and, therefore, a greater efficiency of the antimicrobial function [32].

Synergistic activity with β-lactam antibiotics

β-lactam antibiotics have therapeutic importance in both human and veterinary settings. The synergy observed with oxacillin and meropenem highlights the potential of OESAM as a modulator of antimicrobial activity, an approach that is rarely explored in the scientific literature [33]. The results from the drug interaction tests involving 10 bacterial isolates, including the standard strain *S. aureus* ATCC 25923, showed that the tested antimicrobials exhibited synergistic effects in 50% of cases. This data is relevant because it suggests that combining agents, including EOs and antibiotics, can potentiate antimicrobial activity against resistant strains, representing a promising strategy for combating bacterial MDR. In addition, 27.7% of the isolates showed an additive effect, i.e., the combination of compounds increased antimicrobial activity, although to a lesser extent than synergism. This finding reinforces the importance of investigating antimicrobial associations as an alternative therapy. In contrast, 22.3% of the isolates showed an indifferent response to the combination of compounds, indicating that the action of one compound does not interfere with the action of the other. Taken together, these data show that assessing the interaction profile between bioactive compounds and antibiotics is essential for targeting more effective therapeutic strategies, especially in the context of infections caused by resistant bacteria. The use of tests such as the checkerboard, combined with the calculation of the FICI, is essential for selecting combinations with clinical potential.

Bioinformatic analysis and mechanistic insights

Bioinformatic analysis, as shown in the protein interaction network in Figure 6, is important because protein interaction networks help understand the molecular mechanisms underlying specific conditions [34]. The three clusters observed in the protein interaction network reveal distinct mechanisms of action of WPI 90% on *S. aureus* MDR strains. Clusters are sets of proteins with a greater degree of interconnection within the network and may represent specific molecular, biological, and functional mechanisms [35].

Functional roles of identified protein clusters

With regard to cluster 1, represented by the tryptophan biosynthesis pathway, tryptophan catabolites, such as indole, play an important role in microorganisms, affecting spore formation, plasmid stability, drug resistance,

biofilm formation, and virulence [36]. The antimicrobial activity of this metabolite against *S. aureus* and other bacteria has been reported [37, 38]. In addition, Roager and Licht showed that microbial tryptophan catabolites produced by proteolysis can influence host health, suggesting that these metabolites activate the immune system [39].

Cluster 2, which encodes the siderophore biosynthesis pathway, suggests that *S. aureus* can use this pathway as a resistance mechanism to acquire nutrients, such as iron [40]. Bacteria synthesize siderophores, such as staphylopherrin A and staphylopherrin B, which bind strongly to iron and are recognized by specific membrane receptors, enabling them to assimilate iron-bound complexes [41]. Therefore, by blocking this mechanism, microencapsulation can potentiate the effect of clove EO, which has a strong antioxidant action due to its high eugenol content, which acts as a ferric ion chelator, thereby preventing hydroxyl radical formation [42].

The phosphorelay signal transduction system, defined in cluster 3, provides insight into bacterial signal transduction through regulator phosphorylation [43]. For bacteria such as *S. aureus* to perform their biological functions, the phosphorylation of their response regulators is often necessary. In this sense, any factor that interferes with the phosphorelay signal transduction system can prevent the bacteria from acting [44, 45].

The amino acid biosynthesis pathways, secondary metabolite biosynthesis, and metabolic pathways obtained by analyzing the KEGG pathway of action of WPI 90% revealed that the results obtained in the *in silico* analyses support the hypothesis that the amino acid constituents of WPI 90% can potentiate OESAM action by interfering with these pathways of action of *S. aureus*.

Implications for AMR reduction and future directions

Additionally, the use of OESAM is a therapeutic strategy that can contribute to the reduction of antibiotic residues in the food chain and the environment. These residues, resulting from the intensive use of drugs in farm animals, pose a risk to public health, favoring AMR and potential adverse effects in humans, such as allergic reactions and chronic risks. Therefore, incorporating natural and sustainable alternatives offers a safer and more environmentally responsible approach [46].

The recommended next steps include in vivo investigations, pharmacokinetic evaluations, toxicity studies, and the development of specific formulations for clinical application.

CONCLUSION

This study demonstrated that OESAM markedly enhanced the antimicrobial performance of β-lactam antibiotics against MDR *S. aureus* isolated from bovine mastitis. The microencapsulation process produced structurally stable microcapsules with a yield of 39.11%. Although the encapsulation efficiency was modest, the formulation maintained integrity, physicochemical stability, and protective capacity for the bioactive compounds. The combination of OESAM with oxacillin and meropenem significantly reduced their MICs, with mean decreases of 31 µL/mL and 29 µL/mL, respectively, corresponding to reductions in antimicrobial doses of up to 2.7-fold. FICI values further confirmed synergistic or additive effects in most bacterial strains, highlighting the modulatory potential of OESAM. *In silico* protein interaction analyses revealed three key molecular clusters: tryptophan biosynthesis, siderophore biosynthesis, and phosphorelay signal transduction, suggesting that amino acids present in WPI 90% may contribute to disrupting metabolic and regulatory pathways that support bacterial survival.

The practical implications of these findings are substantial for veterinary medicine and dairy production systems, where the high prevalence of AMR demands alternative strategies for infection control. The ability of OESAM to potentiate β-lactam activity demonstrates its utility as a natural, stable, and economical adjuvant that can reduce antibiotic input, minimize treatment failures, and potentially lower antimicrobial residues in milk and the environment. This represents a promising step toward developing sustainable and residue-free therapeutic approaches aligned with One Health principles.

A major strength of this study is the integration of microbiological, microencapsulation, and bioinformatic analyses, offering mechanistic insights that reinforce the observed *in vitro* effects. However, limitations include the relatively low encapsulation efficiency, the exclusive use of *in vitro* microbial assays, and the lack of physicochemical interaction studies between OESAM and antibiotics. These constraints highlight the need for additional research to improve formulation parameters and validate biological effects in real-world conditions.

Future investigations should focus on optimizing microencapsulation efficiency, assessing stability under industrial or field conditions, evaluating pharmacokinetics and toxicity in animal models, and developing controlled-release formulations suitable for commercial application. Broader antimicrobial panels, molecular

expression studies, and in vivo mastitis models will further clarify the therapeutic potential and safety of OESAM–antibiotic combinations.

In conclusion, this study provides strong evidence that microencapsulated clove EO is a powerful synergistic agent that enhances β-lactam efficacy against MDR S. aureus. By integrating natural bioactives with conventional therapies, this approach offers a promising, innovative, and environmentally responsible strategy to manage AMR in livestock systems and support sustainable animal health solutions.

DATA AVAILABILITY

All the generated data are included in the manuscript.

AUTHORS' CONTRIBUTIONS

ACA, EMSS, IPDO, and CMA: Study design and conception. EMSS, CNDS, HOS, IPDO, and ECGD: Data analysis and drafted the manuscript. AFDNS, CMCC, and HOS: Literature review and drafted the manuscript. ECGD, EMSS, AFDNS, CMCC, and HOS: Conducted laboratory experiments and bioinformatic analysis. ECGD, CMCC, FSADF, and CMA: Collected the samples and data. All authors have read, reviewed, and approved the final manuscript.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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