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Antimicrobial And Antibiofilm Action Of *Eugenia Caryophyllata* Essential Oil Microencapsulated With Whey Protein Isolate

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Abstract:

Background*: The aim of this study was to evaluate the yield and efficiency of microencapsulation, as well as the antimicrobial and antibiofilm activity, of E. caryophyllata essential oil microencapsulated with whey protein isolate and gum arabic.*

Materials and Methods*: We also predicted the mechanism of action of whey protein isolate on the bioactive compound eugenol using bioinformatic analysis. The effects of the microencapsulated clove oil on the strains Staphylococcus aureus ATCC 43300 and Escherichia coli ATCC8739 were evaluated to determine the minimum inhibitory concentration, minimum bactericidal concentration, and adherence of the strains to stainless-steel coupons.*

Results*: When the pH reached 3.75, a layer of coacervates of whey protein isolate whey protein isolate and gum arabic was formed around the oil droplets. The microencapsulated essential oil was 100% effective in reducing E. coli 8739 and S. aureus 43300 adhered to stainless-steel coupons, supporting the efficiency of microencapsulation in whey protein isolate. Bioinformatics analysis revealed an interaction between eugenol and the essential amino acids present in whey protein isolate involving nicotinamide adenine dinucleotide phosphate (NADP) and zinc. Thus, microencapsulation of clove essential oil using whey protein isolate is a promising alternative.*

Key Word: *Microencapsulation; biofilm, bioinformatic analysis; complex coacervation; Staphylococcus aureus; gum Arabic.*

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I. Introduction

The essential oil of *Eugenia caryophyllata* is a medicinally important product rich in eugenol, which has a wide range of effects including antifungal, antibacterial, and antibiofilm activities (Zhang, et al. 2017; Fernandes et al. 2020, Budri et al. 2015). However, this essential oil is hydrophobic; sensitive to external agents such as light, heat, air, and humidity; and is highly volatile, making it difficult to use in the food industry (Botrel et al. 2012). Microencapsulation has been applied in a wide variety of food sectors, such as in the dairy industry, to overcome such limitations (Martín et al. 2015).

Microencapsulation involves trapping an active substance within a coating material. This technique reduces the volatility, hygroscopicity, and reactivity of the compound, increasing its stability under adverse conditions and improving the product's shelf life (Silva et al., 2014; Comunian et al. 2016).

The effectiveness and stability of microcapsules can be influenced by the wall material used for microencapsulation (De Souza et al. 2013). Although numerous polymers can be used as wall materials, only biocompatible and biodegradable materials have been proposed for encapsulation. Gums, lipids, and proteins are frequently used as wall materials (Dubey, 2009).

Because of its ability to adsorb onto lipophilic surfaces, gum arabic (GA) acts as a film-forming agent and protective colloid against the oxidation of volatiles (Kaushik and Roos, 2007). Proteins, in turn, reduce

interfacial tension on the oil-water surface because of their amphiphilic nature (Lam and Nickerson, 2013), making them suitable for encapsulation of hydrophobic compounds.

The use of whey protein to encapsulate the essential oil of *E. caryophyllata* remains limited, and molecular analyses of the interaction between whey protein isolate (WPI) and bioactive compounds in the essential oil are lacking.

The aim of this study was to evaluate the yield and efficiency of microencapsulation, as well as the antimicrobial and antibiofilm activity, of *E. caryophyllata* essential oil microencapsulated with WPI and GA. We also predicted the mechanism of action of WPI on the bioactive compound eugenol using bioinformatic analysis.

II. Material And Methods

Microencapsulation of essential oil (*E. caryophyllata*)

Microcapsules were prepared using a complex coacervation technique (Eratte et al. 2014) with some modifications. Eugenia caryophyllata essential oil was used as the active material, and GA and WPI were used as wall materials. Essential oil (E. caryophyllata) used in this study was purchased from Ferquima (Vargem Paulista, Brazil). It was used in the work of Borborema (2022). We prepared 250 ml of WPI solution (3%) and 250 ml of GA solution (1%) in volumetric flasks. The solutions were magnetically stirred (800 rpm) at temperature the 25ºC for 30 min for complete homogenization. Next, 12.5 g (5%) E. caryophyllata essential oil was added dropwise to the WPI solution. This mixture was stirred using an ultraturrax (T 25 digital ULTRA-TURRAX®, IKA, Campina, Brazil) at 1500 rpm for 10 min. A 250 ml solution of GA (1%) was slowly added to the WPI (3%) + essential solution to obtain a 500 ml solution of WPI + GA + essential oil, which was maintained on a magnetic stirrer.

The pH of this emulsion was adjusted to 3.75 by adding 1% citric acid dropwise to induce electrostatic interactions between WPI and GA. The effects of the pH and capsule morphology were evaluated using an optical microscope. After microcapsules formation, the temperature was reduced to 10°C and the microscopic evaluations were repeated. All processes were conducted under agitation.

The solution was refrigerated at 10ºC for 24 h so that the coacervates could be decanted. After decantation, the supernatant was discarded, and the remaining material was freeze-dried.

Yield and microencapsulation efficiency

To calculate the yield (%) of the microcapsules, the ratio between the final weight (quantity of microcapsules after freeze-drying) and initial weight (quantity of WPI, essential oil, and GA used to prepare the microcapsules) was determined.

Microencapsulation efficiency was calculated by measuring the oil present on the surface and total oil in the microcapsules (Eratte et al. 2014 with modifications). A microcapsule sample (3g) was added to 30 ml of hexane and shaken for 5 min at 225 rpm on an orbital shaker. The solution was filtered through filter paper (5µm), and the retained solid particles were washed three times with 10 ml of hexane. The filtrate was heated to 60ºC on the rotavapor to extract the oil and eliminate hexane. The sample was then placed in an exhaust hood for 4 h to remove residual hexane.

The total oil content was determined using the 4N hydrochloric acid digestion method (Eratte et al. 2014), with modifications. A microcapsule sample (3 g) was added to 30 ml of 4N HCl solution and stirred at 225 rpm for 15 min. Next, 15 ml of hexane was added to the HCl + microcapsule solution and stirred for an additional 18 h at room temperature. The solution was centrifuged (Sorval ST 16R, Thermo Scientific, Waltham, EUA) at 24,471 ×g at 10°C for 30 min. The hexane phase containing the dissolved oil was heated to 60°C on the rotavapor to extract the oil. The sample was placed in an exhaust hood for 4 h to eliminate residual hexane.

The total oil, surface oil, and microencapsulation efficiency were calculated according to the following equations:

Suface oil (%) = (surface mass of microcapsules (MS) / weight of microcapsules) x100

Total oil (%) = (total mass of microcapsules (MT)/ weight of microcapsules) x100

Microencapsulation efficiency (%) = (MT- MS/MT) x100

where MS and MT are the surface mass (g) and total mass of the microcapsules (g), respectively.

Minimum inhibitory concentration and minimum bactericidal concentration

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of microencapsulated E. caryophyllata essential oil were determined using a microdilution test as described by NCCLS (2003) with some modifications.

Pure bacterial cultures of Staphylococcus aureus ATCC 43300 and Escherichia coli ATCC8739 were grown in brain heart infusion (BHI) broth for 24 h at 37°C and then standardized according to the Mcfarland scale at score 0.5 (1.5 × 108 CFU/mL) according to CLSI (2015).

Microencapsulated E. caryophyllata essential oil was tested for its antimicrobial effects at concentrations of 360, 180, and 90µl/ml to determine the MIC and MBC. The efficacy of the microcapsules was determined by adding 90µL of the microencapsulated oil solution, 90µL of BHI + Tween 80 broth (0.1%), and 10µL suspension

of the microorganism to be tested to the wells of the microplate. The plate was then incubated for 24 hours at 37°C, in BOD (Biochemical Oxygen Demand). After which 10µl triphenyltetrazolium chloride was added and the plates were further incubated again for 2 h (Duarte et al. 2005). The wells showing bacterial growth were colored red, whereas those without growth were colorless.

As a positive control, 10µl of the microorganism (E. coli and S. aureus) and 180µl of BHI + Tween 80 broth (0.1%) were used. The negative control contained 180µl of BHI broth + Tween 80 (0.1%). Non-microencapsulated essential oil was also used as a control (20µL) based on the results of Borborena (2022).

Biotransfer of *E. coli* and *S. aureus* to stainless steel coupons

The strains showing an MBC in the presence of microencapsulated essential oil were used to evaluate biotransfer test on stainless steel coupons. The bacteria were activated in BHI broth and standardized in a spectrophotometer at 570 nm to an optical density value of 0.5.

The method used to evaluate the biotransfer potential was described by Malheiros et al. (2010). The tested strains were placed in contact with the stainless-steel coupons for 48h at 36°C. The coupons were then subjected to the following treatments: immersion in microencapsulated oil solution (concentration 180µl for S. aureus ATCC 43300 and 90µl for E. coli ATCC8739); immersion in non-microencapsulated essential oil at a concentration of 20µl, immersion in the positive control solution with the tested strains and negative control with sterile distilled water. Using sterile tweezers, the coupons were separately immersed in 10 ml of 0.85% (w/v) NaCl to remove planktonic (weakly adhered) cells. They were then immersed in the test solutions, and the sanitizing action was evaluated after 30 min of contact under static conditions at a temperature of 25 ± 2°C. The coupons were transferred to 10 ml of 0.85% (w/v) saline solution and sonicated for 2 min in an ultrasonic bath (GS-DS2P240, Allcrom, São Paulo, Brazil) at 40 kHz to remove adhered cells that survived on the coupon surface (Malheiros et al. 2010). Successive decimal serial dilutions of the treatments were prepared and inoculated on plates containing MacConkey agar for treatment with E. coli ATCC 8739 and mannitol salt agar for S. aureus ATCC 43300. The plates were incubated at 37°C for 48 h. The results were evaluated as described by Careli et al. (2009) using the following equation.

Number of adhered cells:
CFU/cm2 = count (CFU/ml) × volume of diluent used to remove adhered cells (ml) / area of coupons (cm2)

Bioinformatic analysis

The interactions between eugenol, the main active ingredient in clove essential oil, and whey proteins used in the microencapsulation wall were investigated using the STITCH database (version 5.0; http://stitch.embl.de/), as described by Kuhn et al. (2012). The platform was used to search for chemical-protein interactions in Bos taurus, inserting the essential amino acids present in the WPI formulation and chemical compound eugenol as input descriptors. The overall STITCH score, with an average confidence of 0.4, was used as a measure of association, with no more than 10 interactors in the first layer and a maximum of 60 interactors in the second layer.

A chemical-protein interaction subnetwork was created with the same average confidence from eugenol and direct ligands of the molecule showing a direct interaction with eugenol in the first network formed. The active interaction sources used in the network and subnetwork were mining, experiments, databases, co-expression, neighborhood, gene fusion, co-occurrence, and prediction. Network and subnetwork analyses were based on the association of the chemical compound eugenol with other compounds present in WPI. A table was downloaded from the STITCH database to show the combined scores of the interactions between the molecules.

Experimental design and data analysis

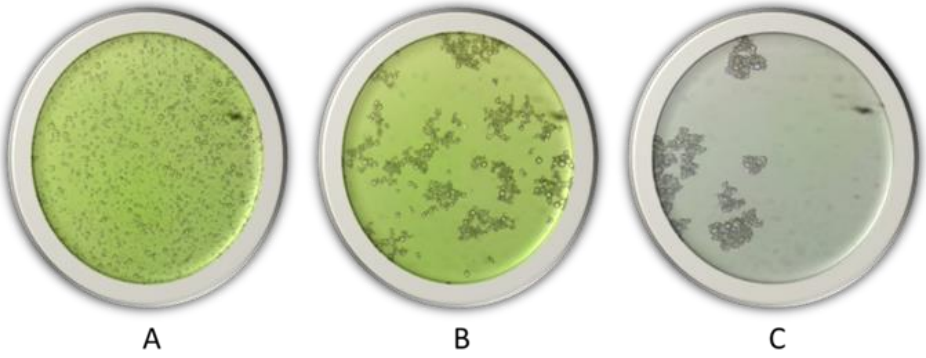
To assess the inhibitory effect of microencapsulated essential oil on biofilm formation, a completely randomized design was used with four treatments (Positive control, Negative control, Microencapsulated E. caryophyllata essential oil and E. caryophyllata essential oil) and four replicates. The microbial count data (CFU) were expressed in log form and evaluated using analysis of variance at a 5% probability level. The means were compared using Tukey's test ($p < 0.05$) and analyzed using Studio R software.

III. Result And Discussion

At the start of microcapsule formation, the initial pH was 6.0 and the oil droplets were dispersed in the solution (Fig. 1A). When citric acid (1%) was gradually added, the pH began to decrease; when the pH reached 4.7, the oil droplets began to aggregate (Fig. 1B). According to Eratte et al. (2014), this aggregation may be related to the weaker repulsion of WPI and GA when the WPI charge approaches neutrality. When the pH was below 4.7, aggregation between the oil droplets increased. According to Eratte et al. (2014), complex coacervation

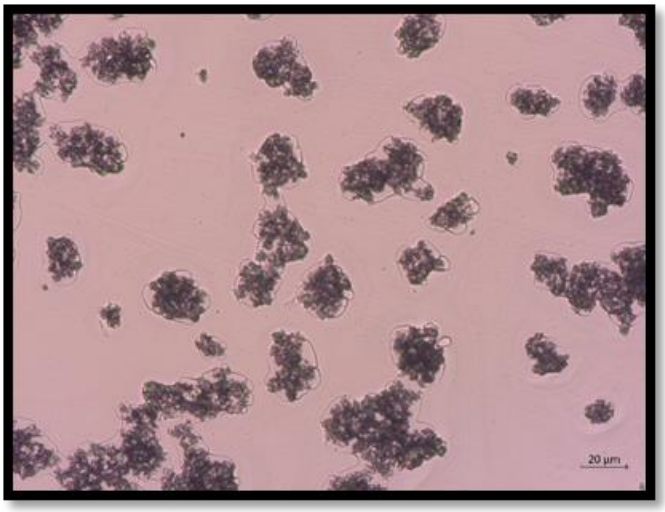
begins when the pH of the dispersion is reduced to below the isoelectric point of WPI (4.4) (Fig. 1C) because WPI became positively charged and electrostatic attraction occurred with negatively charged GA.

Figure.1 Coacervation process observed through optical microscopy. (A) pH 6.0; (B) pH 4.7; (C) pH 4.2



When the pH reached 3.75, a layer of WPI-GA coacervated around the oil droplets (Fig.2). The average yield of the microcapsules was 45.03%. Lima et al. (2023) showed that the yield of the spray drying microencapsulation process was 54%. Justi (2017) reported a yield of 67% for the formation of microcapsules through a complex coacervation process. The concentration of oil retained in the microcapsules can be influenced by the surface area of the materials during spraying, diffusion of volatile substances when the capsule-forming membrane is not instantaneous, and transfer of volatile materials due to the formation of bubbles during the drying process (Felix et al., 2017). According to Siow et al. (2013), when one of the encapsulating agents (WPI or GA) is in excess in the system, the presence of non-neutralized charges reduces the attraction between the two colloids, thus reducing the yield of coacervates.

Figure. 2 Formation of microcapsules (pH 3.75) using optical microscopy. Scale: 20µm



The concentration of 90µl/ml of microencapsulated oil showed an MIC for *S. aureus* ATCC 43300. The same concentration exhibited a bactericidal effect (on *E. coli* ATCC 8739. When the concentration was increased to 180µl, there was a bactericidal effect for *S. aureus* ATCC 43300. These results helped to establish the ideal concentration for biofilm analysis. According to Monteiro (2023), use of microencapsulated lemongrass essential oil showed an MIC and MBC of 250µg/ml for *E. coli* and 125µg/ml *S. aureus*, respectively. Our results differed from the MIC and MBC values described in the literature. Essential oils differ in their composition according to external factors, such as the area and growing conditions of the plant, genetic modifications, climatic conditions, parts of the plant, growth stages, and harvest time. In addition, the oil extraction methods must be considered. Thus, these factors significantly interfere with the composition of the essential oil and, consequently, its antimicrobial activity (Türkmen 2021).

The results showed that the microencapsulated essential oil was 100% effective in reducing *E. coli* 8739 and *S. aureus* 43300 to the stainless-steel coupons (Table 1). The non-microencapsulated essential oil showed similar results as those of non-microencapsulated oil.

Table 1- Number of cells (log CFU/cm2) of *Escherichia coli* and *Staphylococcus aureus* strains on stainless steel surfaces after treatment with microencapsulated rosemary essential oil (OM) and clove essential oil without microencapsulation (OE) for an exposure time of 30 min at 25 ± 2 °C.

Treatments	***S. aureus 43300***	***E. coli 8739***
CP	6,205 a	4,885 a
CN	0,000 b	0,000 b
OM	0,000 b	0,000 b
OE	0,000 b	0,000 b
CV	2,56	6,17

Positive control (PC); Negative control (NC); Microencapsulated clove oil (OM); Clove essential oil (OE)

Similar results were found by Borges et al. (2021), where treatments with solutions of plant extracts of *Calophyllum brasiliense* and *Annona crassiflora* reduced *E. coli* 8739.

Boari et al. (2009) established a value of 10^5 CFU/cm^2 to differentiate between the adhesion process and a biofilm, as this value is intermediate that proposed by Bridgeman and Zottola (1998), who established a minimum surface area of 7 log CFU/cm^2, and Wirtanen, Husmark, and Mattila-Sandholm (1996) and Ronner and Wong (1993), who considered 10^3- 10^5 adhered cells per cm^2 as a biofilm.

Staphylococcus aureus 43300 and *E. coli* 8739 formed a biofilm in the control treatments. Although biofilm formation was not verified, adhesion was assumed because adhered bacteria are more resistant to sanitizers than are those in suspension (Borges et al. 2021).

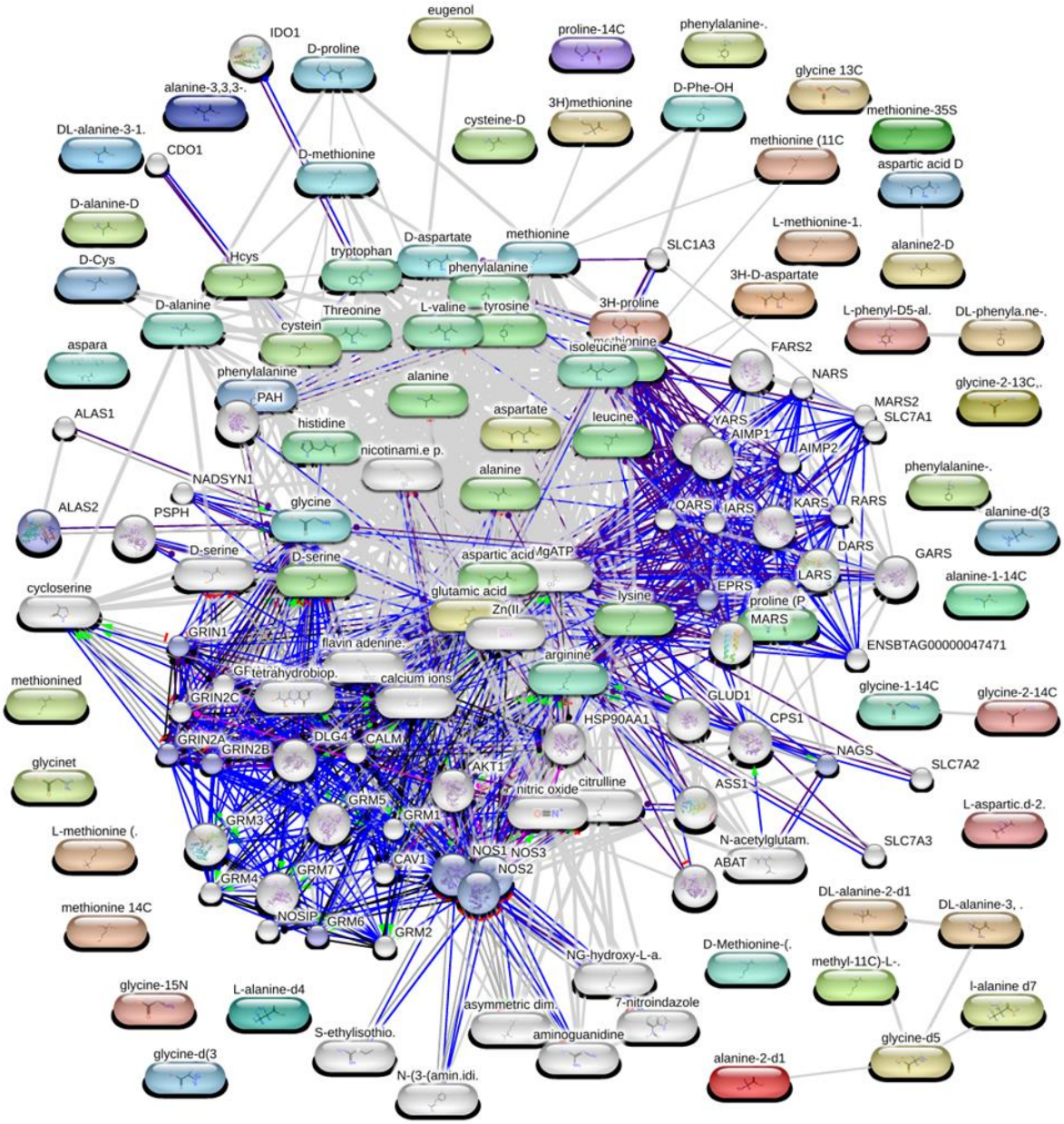
Similar efficiencies between encapsulated and non-encapsulated oils indicate promising prospects for the use of the encapsulation technique, owing to the increase in the useful life of the compounds and the control of their release into the environment. Encapsulation protects bioactive compounds from factors that can cause them to deteriorate, such as light and oxygen, thereby contributing to product stability over prolonged periods (Desai and Park, 2005).

Bioinformatics analysis can improve the understanding of the effects of WPI, used as a wall material, on the bioactive compound eugenol. Eugenol (4-allyl-2-methoxyphenol) is a volatile phenolic compound that is the main constituent of oils extracted from cloves (Mazzafera 2003). Its concentration varies according to the part of the plant analyzed and region in which it is grown. In the leaves, eugenol accounts for approximately 95% of the extracted oil in the leaves and 70–85% in the flower bud as the main component (Affonso et al. 2012; Mazzafera 2003).

The functional properties of whey proteins make them interesting candidates as wall materials in the manufacture of microparticles, including their solubility, emulsifying capacity, emulsion stability, and microparticle-forming capacity. In addition to their encapsulation properties, whey proteins are an excellent source of essential amino acids, which favors their applicability (Abbasi et al. 2014). Figure 3 shows the results of network analysis, which demonstrates the interaction between eugenol and the essential amino acids present in WPI. This interaction occurs through nicotinamide adenine dinucleotide phosphate (NADP), a coenzyme of nicotinamide (Jacob 2006). Nicotinamide is essential in the form of the coenzymes NAD+/NADH and NADP+/NADPH, with niacinamide functioning as an electron acceptor in catabolic reactions or as an electron donor in anabolic reactions (Berdanier 1998; Jacob 2006). The eugenol biosynthesis reaction is dependent on NADP (Simões, Spitzer, 2001), the coenzyme form of nicotinamide, which is produced from biological conversion of tryptophan (Jacob, 2006), an essential amino acid present in the WPI used in this study. In addition, a balance of essential amino acids is necessary for satisfactory biosynthesis of nicotinamide (Patterson et al. 1980). Therefore, use of WPI as a wall material in the encapsulation process may improve the stability of the bioactive compound eugenol.

In addition, eugenol is classified as a phenylpropanoid formed from the aromatic amino acid phenylalanine, a substance formed in the shikimate/aragonate pathway (Ferrer et al. 2008) and is present in 2.72% of the WPIs. The shikimic acid route converts carbohydrate precursors derived from glucose and pentose phosphate into aromatic amino acids. The formation of many plant phenolic compounds, including simple phenylpropanoids, coumarins, benzoic acid derivatives, lignin, anthocyanins, isoflavones, condensed tannins, and other flavonoids, begins with phenylalanine (Taiz and Zeiger, 2004).

Figure 3 - Chemical-protein interaction network based on eugenol and amino acids present in whey protein isolate (STITCH, 2024)



The chemical-protein interaction subnetwork shown in Figure 4 was drawn from the descriptors eugenol, NADP, and direct NADP ligands in the network shown in Figure 3. In addition to its interaction with NADP, eugenol interacts with zinc and the essential amino acids present in WPI. The interaction between zinc oxide and eugenol is triggered in the presence of water and consists of a chelating reaction that results in the formation of a zinc eugenolate matrix with unreacted zinc oxide particles and a small amount of free eugenol. This is a reversible reaction; in the presence of water, eugenolate undergoes hydrolysis and releases more eugenol (Bauer et al. 2008). In addition, zinc plays important roles, particularly as an enzyme activator, and is required for the synthesis of essential amino acids such as tryptophan (Mengel and Kirkby,1987).

These findings provide evidence that WPIs contribute to the stability of eugenol, which is the main bioactive compound in clove oil.

Figure 4 - Chemical-protein interaction subnetwork made from eugenol, NADP and direct NADP ligands (STITCH, 2024).

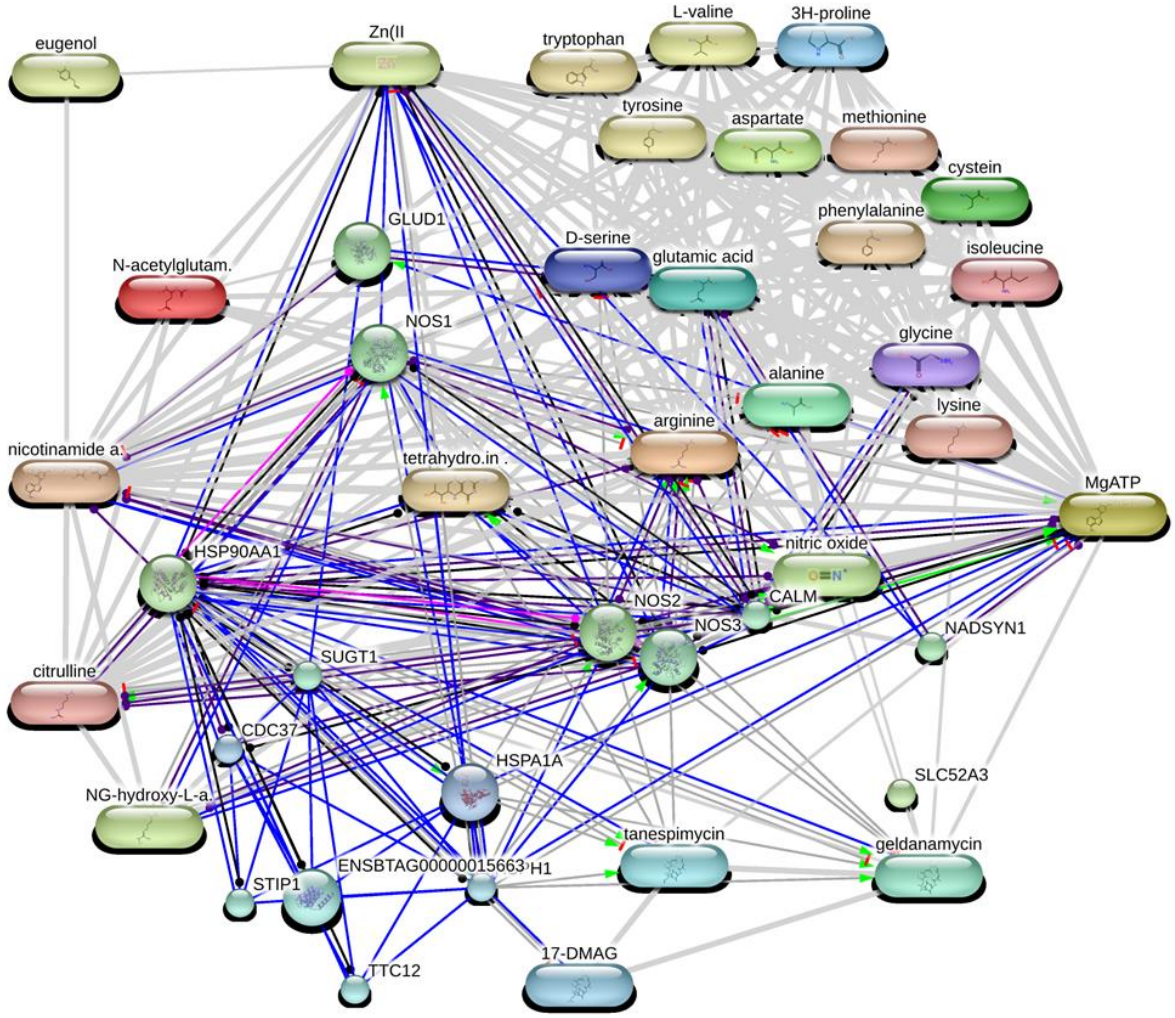


Table 2 shows the combined interaction scores between the different molecules extracted from the network and subnetworks in Figures 3 and 4, respectively. The combined scores for the association of eugenol with NADP and zinc were 0.80 and 0.45, respectively.

Table 2 - Combined interaction score between different molecules with eugenol and essential amino acids present in whey protein isolate.

Node 1	Node 2	Combined score	Node 1	Node 2	Combined score
nicotinamide a.	Zn	0.920	methionine	nicotinamide a.	0.920
nicotinamide a.	tyrosine	0.910	alanine	Zn	0.965
nicotinamide a.	phenylalanine	0.909	arginine	Zn	0.968
nicotinamide a.	tryptophan	0.907	aspartate	Zn	0.963
nicotinamide a.	L-valine	0.900	cystein	Zn	0.955
nicotinamide a.	eugenol	0.800	D-serine	Zn	0.927
3H-proline	nicotinamide a.	0.900	eugenol	Zn	0.450
alanine	nicotinamide a.	0.921	glutamic acid	Zn	0.966
arginine	nicotinamide a.	0.953	glycine	Zn	0.705
aspartate	nicotinamide a.	0.945	isoleucine	Zn	0.432
cystein	nicotinamide a.	0.919	lysine	Zn	0.971
D-serine	nicotinamide a.	0.900	methionine	Zn	0.971
glutamic acid	nicotinamide a.	0.919	nicotinamide a.	Zn	0.920
glycine	nicotinamide a.	0.920	phenylalanine	Zn	0.947
isoleucine	nicotinamide a.	0.900	tyrosine	Zn	0.927
lysine	nicotinamide a.	0.927	-	-	-

The *in vitro* and *in silico* results indicated promising prospects for the use of WPI as a wall material for microencapsulation of clove essential oil. Encapsulation with milk proteins has great potential for the production of microencapsulated probiotics (Kim et al., 2017). Whey proteins have almost all essential amino acids in large quantities, except for aromatic amino acids (phenylalanine, tyrosine). They also contain high concentrations of the amino acids tryptophan, cysteine, leucine, isoleucine, and lysine (Sgarbieri, 2004). The interactions between eugenol and the amino acids may contribute to the performance and stability of eugenol.

IV. Conclusion

Microencapsulated clove essential oil in WPI and GA as wall materials showed antimicrobial activity against strains of S. aureus 43300 and E. coli 8739. The microencapsulated oil showed antibiofilm activity against S. aureus 43300. Although E. coli 8739 is not considered a biofilm-forming strain, the use of microencapsulated oil reduced the adhesion process. Therefore, microencapsulation of clove essential oil is a promising alternative for increasing the shelf-life of the product. Its main compound is eugenol, which is likely responsible for its antimicrobial and antibiofilm activities. Bioinformatics analyses indicated that eugenol's activities are favored when combined with WPI through interactions with essential amino acids, mediated by NADP and zinc. Functional studies are required to confirm the synergistic effects of eugenol and essential amino acids.

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