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Clove and Thyme Essential Oils: From Molecular Docking to Food Application—A Study of Their Preservative Properties in Buttermilk

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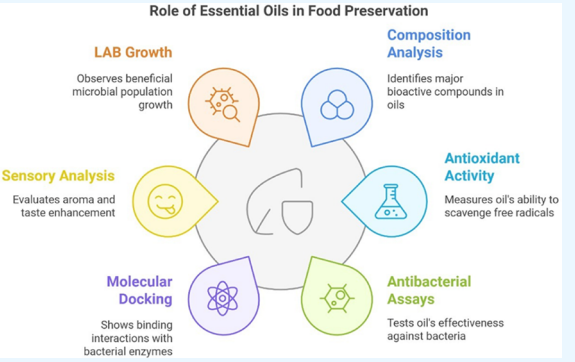
ABSTRACT: This study investigates clove (CEO) and thyme (TEO) essential oils as natural preservatives, focusing on their composition, antimicrobial and antioxidant properties, and application in buttermilk. In the first part, GC-MS analysis identified eugenol (73.45%) and thymol (27.53%) as the major bioactive compounds in CEO and TEO, respectively. Antioxidant activity assays revealed strong performance for CEO, with EC_{50} values of 0.058 mg/mL for H_2O_2 scavenging and 0.063 mg/mL for DPPH, significantly outperforming TEO (EC_{50} values of 0.102 and 0.106 mg/mL, respectively). In vitro antibacterial assays demonstrated CEO's superior efficacy, achieving minimum inhibitory concentrations (MICs) as low as 25 mg/L against Gram-positive bacteria and 50 mg/L against Gram-negative bacteria, while TEO exhibited MICs ranging from 50 to 100 mg/L. Molecular docking highlighted selective binding of eugenol (−6.5 kcal/mol) and thymol (−5.9 kcal/mol) to bacterial enzymes, underpinning their selective antimicrobial mechanisms. In the second part, buttermilk was fortified with CEO and TEO, and sensory analysis revealed that TEO significantly enhanced aroma and taste, achieving a mean score of 7.93 for taste at 100 μg/mL, while CEO exhibited a more neutral sensory impact with a mean score of 6.14 at the same concentration. Additionally, CEO and TEO supplementation promoted LAB growth, sustaining beneficial microbial populations over a 5-day storage period and preserving microbiological quality comparable to untreated samples. These findings highlight CEO and TEO as effective natural preservatives for functional food systems, combining selective antimicrobial, antioxidant, and sensory benefits.



INTRODUCTION

In recent years, consumers have increasingly demanded safer, healthier, and more sustainable food products, with a particular emphasis on natural alternatives to synthetic preservatives.[1] This trend reflects heightened consumer awareness of the potential health risks associated with chemical additives and a preference for clean-label food products. To meet this demand, researchers and food manufacturers are exploring innovative preservation methods that prioritize both food safety and quality while aligning with consumer expectations for natural ingredients.[2−5] Among the promising solutions are plant-derived essential oils (EOs), which have gained attention for their potent antimicrobial, antioxidant, and therapeutic properties.[6,7] EOs are complex mixtures of secondary metabolites, underscoring their potential as multifunctional agents in food preservation.[6,7]

EOs, particularly clove (CEO) and thyme (TEO) essential oils, have been extensively studied for their ability to inhibit the growth of pathogenic microorganisms while enhancing the sensory and nutritional qualities of food products.[8−13] These properties have positioned EOs as valuable additives in dairy applications, where they not only extend shelf life but also boost organoleptic properties and nutritive potential.[8−13]

Dairy products, especially fermented ones like buttermilk, have a unique nutritional and functional profile, offering benefits such as lactose digestion improvement, immunomodulatory effects, and cholesterol management.[14,15] However, maintaining the balance between beneficial microorganisms, such as lactic acid bacteria (LAB), and inhibiting spoilage organisms remains a challenge in dairy preservation.[16] LAB, including *Lactobacillus acidophilus* and *Bifidobacterium bifidum*, are essential for the development of probiotic dairy products, contributing to therapeutic effects and enhancing product

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functionality.[14,17] The selective inhibition of pathogenic bacteria while preserving LAB activity is therefore a critical requirement in the development of effective natural preservatives for dairy products.

The complex flavor profile of dairy products arises from volatile acids, carbonyl compounds, and amino acids, which are often complemented by natural flavoring agents such as EOs.[18,19] Moreover, the antioxidant properties of EOs, which include scavenging free radicals and inhibiting lipid peroxidation, further support their role in extending the shelf life and stability of food systems.[18,20,21]

Despite the extensive research on EO efficacy, the integration of computational approaches, such as molecular docking, with practical food applications remains underexplored. Molecular docking provides critical insights into the interactions between EO-derived bioactive compounds and bacterial enzymes, shedding light on the mechanisms of selective inhibition of spoilage organisms without compromising beneficial microbiota. This mechanistic understanding is particularly relevant for dairy products like buttermilk, where preserving LAB activity is crucial for maintaining functional and sensory properties.

In this study, we investigate the potential of CEO and TEO as dual-function preservatives and flavorants in buttermilk. By combining experimental evaluation of their antioxidant and antimicrobial activities with sensory analysis and shelf life assessment, this work provides a comprehensive exploration of their suitability for application in dairy products. The novelty of our study lies not only in the specific focus on buttermilk but also in the use of in silico docking to elucidate the molecular mechanisms underlying their selectivity against pathogenic and beneficial bacteria. This mechanistic insight, combined with the practical assessment of shelf life and sensory properties, distinguishes this research from previous studies that have primarily focused on EO efficacy in other food systems.

MATERIALS AND METHODS

Materials. *Essential Oils, Chemicals, and Media.* Commercial 99.8% purity food-grade EO were used of *Thymus vulgaris* (purchased from NPF Zarstvo Aromatov, Sudak, Ukraine) and Clove (*Syzygium aromaticum* L. Myrtaceae) purchased from Aroma Inter, Mykolaiv, Ukraine; Aromatika, Kiyiv, Ukraine; NPF Zarstvo Aromatov, Sudak, Ukraine). Solvents, DPPH, and ABTS from Sigma- Aldrich (Germany). Culture media Brain Heart Infusion broth (BHI), de Man, Rogosa, and Sharpe broth (MRS), and Nutrient Agar (NA) sourced from Oxoid, UK.

Bacterial Strains. The bacterial strains utilized in this study were sourced from two reputable microbial culture collections to ensure the accuracy and reliability of the experimental results. The Lactic Acid Bacteria (LAB) strains, including *Lactobacillus delbrueckii* subsp. *bulgaricus* (DSM 20081) and *Streptococcus thermophilus* (DSM 20617), were procured from the DSMZ (German Collection of Microorganisms and Cell Cultures). Additionally, the *Lactococcus lactis* subsp. *lactis* (ATCC 11955) and all pathogenic and spoilage bacteria strains, such as *Listeria monocytogenes* (ATCC 7644), *Staphylococcus aureus* (ATCC 29213), *Escherichia coli* (ATCC 25922), were obtained from the ATCC (American Type Culture Collection).

Buttermilk. Full pasteurized buttermilk samples (1.00 L each) were obtained from the local market for this study. Based on the information provided on product label, the buttermilk was reported to contain 8.6 g of total fat, 11.6 g of total carbohydrates, and 8.0 g of protein.

Methods. *Hydrogen Peroxide (H_2O_2) Scavenging Activity.* The hydrogen peroxide scavenging activity of the samples was determined following the method of Ruch.[22] A reaction mixture was prepared by adding 1 mL of 30 mM hydrogen peroxide solution to sample solutions at concentrations ranging from 20 to 100 ppm, resulting in a total reaction volume of 3 mL. The absorbance of hydrogen peroxide was measured at 230 nm within 3 min against a blank solution containing ethanol without hydrogen peroxide. The percentage of scavenging activity was calculated using the formula:

$$\%\ \text{Scavenging Activity} = \left(\frac{A_s}{A_c} - 1\right) \times 100$$

where $\mathbf{A_s}$ and $\mathbf{A_a}$ represent the absorbances of the sample and control, respectively.

DPPH Radical Scavenging Activity. The antioxidant activity of the samples was assessed using the DPPH radical scavenging assay, as described by Hatano et al.[23] A reaction mixture was prepared by adding 0.1 mL of 5 mM DPPH solution to sample solutions at concentrations ranging from 20 to 100 ppm, resulting in a total reaction volume of 3 mL. After a 10 min reaction period, the absorbance was measured at 515 nm against a blank solution containing ethanol. The percentage of DPPH radical scavenging activity was calculated utilizing the formula:

$$\%\text{Scavenging Activity} = \left(\frac{A_c - A_s}{A_c}\right) \times 100$$

where A_s and A_a represent the absorbances of sample and control, respectively. The EC_{50} value, which represents the effective concentration required to reduce the initial DPPH concentration by 50%, was calculated. The antioxidant activity index (AAI) was further determined following Scherer and Godoy:[24]

$$\text{AAI} = \frac{\text{DPPH}(\mu\text{g/mL})}{\text{EC}_{50}(\mu\text{g/mL})}$$

Samples were categorized based on their AAI values as poor (AAI < 0.5), moderate (0.5 < AAI < 1.0), strong (1.0 < AAI < 2.0), or very strong (AAI > 2.0).

ABTS Radical Cation Scavenging Activity. The ABTS assay was conducted to assess the ability of the samples to scavenge the ABTS radical cation ($ABTS^+$), following the method of Re.[25] The radical cation was generated by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate in equal volumes and allowing the mixture to incubate in the dark at room temperature until the absorbance stabilized. The ABTS solution was diluted with ethanol to achieve an absorbance of approximately 0.7 at 734 nm. For the assay, 0.9 mL of the $ABTS^+$ solution was mixed with 0.1 mL of the sample, and the absorbance was measured at 734 nm after 1 min. The percentage of scavenging activity was calculated utilizing the formula:

$$\%\ \text{Scavenging Activity} = \left(\frac{A_c - A_t}{A_c}\right) \times 100$$

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where A_t and A_a represent the absorbances of the tested sample and control, respectively. Standards such as BHT and vitamin C were used for comparison.

Qualitative Analysis of Essential Oils via GC-MS. The essential oils of clove and thyme were reconstituted in 1 mL of methanol: diethyl ether 7:3 ratio and 1 μL of sample solution (100 μg EO/ul) injected to separation by GC-MS (Shimadzu-QP-2010S plus) instrument equipped with [AOC-20i+s] autosampler autoinjector and a capillary column (Rtx-1 30 m × 0.32 mm I.D., 0.25 μm). The oven temperature was adjusted for an initial temperature of 70 °C followed by a 10 °C/min temperature ramp to 230 °C and hold for 1 min then raised to 280 °C with 15 °C/min. The final temperature was maintained for 1 min. Injector and mass interface temperatures are adjusted at 280 °C. Helium carrier gas (He) column flow 2.62 mL/min with linear velocity 58.7 cm/sec. The mass parameters were set as following: ion source temp. 220 °C, solvent cut time 4.0 min, MS detector (EI−mode) the compounds were acquired by scan mode ACQ start *m*/*z* 70 and End m/z600. The integration was performed by Lab Solution software 4.1 and compounds were compared by NIST 11s library and the related compounds in literature.

In vitro Evaluation of Antibacterial Activity. Antibacterial activity of clove and thyme essential oils was evaluated against both pathogenic and beneficial bacterial strains (Table 1). The

Table 1. Indicator Bacteria Used in the Study

Code	Strain No.	Bacteria
	Lactic Acid Bacteria (LAB)	
LB81	DSM 20081	*Lab. delbrueckii* subsp. *bulgaricus*
LL55	ATCC 11955	*Lactococcus lactis* subsp. *lactis*
ST17	DSM 20617	*Streptococcus thermophillus*
	Pathogenic and Spoilage Bacteria	
LM44	ATCC 7644	*Listeria monocytogenes*
SA13	ATCC 29213	*Staphylococcus aureus*
SS05	ATCC 15305	*Staphylococcus saprophyticus*
EC22	ATCC 25922	*Escherichia coli*
PA53	ATCC 27853	*Pseudomonas aeruginosa*
SE76	ATCC 13076	*Salmonella enterica*

tested strains included Gram-positive pathogens (*Staphylococcus aureus* SA13, *Staphylococcus saprophyticus* SS05, *Listeria monocytogenes* LM44) and Gram-negative pathogens (*Escherichia coli* EC22, *Pseudomonas aeruginosa* PA53, *Salmonella enterica* SE76). Beneficial lactic acid bacteria (LAB) strains tested were *Lactobacillus plantarum* ST17, *Lactobacillus brevis* LL55, and *Lactobacillus casei* LB81.

Pathogenic bacteria were cultured in Brain Heart Infusion (BHI) broth (Oxoid, UK) at 37 °C for 24 h, while LAB strains were propagated in de Man, Rogosa, and Sharpe (MRS) broth (Oxoid, UK) at 37 °C for 48 h. Working cultures were maintained on respective agar slants at 4 °C.

Agar Well Diffusion Assay for Antibacterial Activity. The antibacterial activity of CEO and TEO was assessed using the agar well diffusion method according to Nzeako et al.[8] Sterile Nutrient Agar (Oxoid, UK) was tempered at 48 °C, seeded with 100 μL of bacterial suspensions (10^t CFU/mL), and poured into Petri dishes. Wells (6 mm in diameter) were made in the agar using a sterile cork borer, and 100 μL of the essential oils at different concentrations were added to each well. Plates were incubated at 37 °C for 24 h, and the diameter of the inhibition zones was measured in millimeters. Inhibition zones greater than 8 mm were considered active.

Minimum Inhibitory Concentration (MIC) Determination. MIC was determined as the lowest concentration of essential oil that produced an inhibition zone greater than 8 mm in agar well diffusion assay according to Preuss e al.[26] Both oils were tested at concentrations of 10, 20, 40, 80, 100 mg/L. For each bacterial strain, MIC values were calculated separately for CEO and TEO.

Selectivity Index (SI) Calculation. Selectivity Index (SI) was calculated to compare specificity of CEO and TEO in inhibiting pathogenic versus beneficial bacteria according to Nunes et al.[27] SI was defined as the ratio of the average MIC for LAB strains (beneficial bacteria) to the average MIC for pathogenic strains. A higher SI indicates greater selectivity for inhibiting pathogens over LAB. The SI values for CEO and TEO were computed using the standardized MIC data.

In Silico Molecular Docking Analysis of Key Compounds. The 3D structures of the three major compounds (ligands) were retrieved from the PubChem database and minimized using Avogadro software. To prepare the ligands for docking, Open Babel software (Version 3.1.1) was used to convert the structures from SDF to PDBQT format. The target bacterial enzyme sequences were either obtained from the Protein Data Bank (PDB) or modeled using the SWISS-MODEL server (https://swissmodel.expasy.org) or enzymes without experimentally determined structures.

Specific enzymes included catalase (PDB ID: 8j4q) and esterase (PDB ID: 3bf8) from *Escherichia coli*, catalase (PDB ID: 4e37) and esterase (PDB ID: 3d7r) from *Pseudomonas aeruginosa*, catalase (template: 1e93.1A), phosphatase (template: 1nw.1A), and protease (template: Q2FxW5.1A) from *Staphylococcus aureus*, catalase (template: 2j2m.1A), phosphatase (template: A0A7 × 1DP51.1A), esterase (template: A0A7 × 0WIF7.1A), and protease (template: A0A464QMC4.1A) from *Listeria monocytogenes*, and alkaline phosphatase (template: 2w8d.1A) from *Lactiplantibacillus plantarum*. Other enzymes included amylase (template: 5z0u.1A), protease (template: A0A5J0S815.1), esterase in *Pseudomonas aeruginosa* (template: Q91644.1A), phosphatase (template: Q9HZ40.1A), and protease (template: Q91417.1A). All modeled enzyme structures were energy-minimized using the Chiron server. The target enzymes were prepared by removing water molecules and other ligands and adding polar hydrogen atoms to the amino acid residues. The PDBQT files for the target enzymes were generated using the AutoDock Tools suite, and the active sites were predicted using the CASTp server. Docking studies were performed using AutoDock Vina according to Trott[28] to evaluate the binding affinity of the ligands to the bacterial enzymes.

The ligands were optimized and docked into the active sites of the enzymes with grid boxes centered at coordinates (12.6, 0.34, 38) for *Listeria monocytogenes*, (−5.2, 44.6, 43.57) for *Escherichia coli*, (40.1, −4.9, 125) for *Lactiplantibacillus plantarum*, (4.7, 12.7, 6.9) for *Pseudomonas aeruginosa*, and (90, 90, 89.9) for *Staphylococcus aureus*. These grid boxes were defined to fully cover the ligand-binding region, and binding affinities were assessed for all enzyme-ligand complexes. Visualization of molecular interactions between the ligands and the target enzymes was conducted using PyMOL software, highlighting key interactions such as hydrogen bonding and hydrophobic interactions. This process allowed for the identification of binding poses and confirmation of ligand

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affinity for the active sites, as described by Angarita-Rodríguez et al.[29] and Sharifinia et al.[30]

In Situ Experimental Design for Fortified Samples. The study was conducted as a completely randomized design with three replicates per treatment group to evaluate the impact of clove and thyme essential oils on shelf life, in situ antibacterial activity, and sensory properties of fortified buttermilk. In both assessments, the oils were incorporated into buttermilk under sterile conditions to ensure consistency and reliability of results. The EOs were added at concentrations of 50 μg and 100 μg per 100 g of milk for the antimicrobial analysis, while sensory evaluation used a concentration of 100 μg per 100 g of milk. The fortified buttermilk samples were aseptically transferred into 250 mL transparent PET bottles and stored at 4 °C ± 1 °C. A negative control (without EOs) and a positive control (Amoxicillin at 100 μg per 100 g of milk) were included to provide comparative benchmarks. Analyses were conducted at intervals of 0, 2, and 5 days.

Sensory Evaluation. Sensory evaluation was conducted to assess the organoleptic properties of EO-fortified buttermilk. A panel of ten trained judges (8 male and 2 females, average age 38 years) evaluated the samples in a well-ventilated and illuminated sensory laboratory at 20−25 °C. Samples were assessed for color, taste, smell, and overall acceptability using a 9-point hedonic scale. Unsalted bread and distilled water were provided for palate cleansing between samples.[31] Before the evaluation, panelists were screened to ensure they had no allergies or sensitivities to any of the essential oil components used in the study.

Evaluation of Antibacterial Activity. For the shelf life assessment, microbial growth was monitored by isolating lactic acid bacteria (LAB) and enumerating spoilage bacteria. Serial dilutions were prepared by adding 10 mL of fermented milk to 90 mL of sterile distilled peptone water (0.1%) and diluting up to 10^{-7}. From the last three dilutions (10^{-5}, 10^{-6}, and 10^{-7}), 0.1 mL was plated on predried Lactobacilli MRS agar plates and incubated anaerobically in a CO_2 incubator at 37 °C with 5% CO_2 for 48 h. Distinct colonies were subcultured onto fresh MRS agar, incubated for 24−48 h, and confirmed as LAB through Gram staining and catalase tests, with Gram-positive and catalase-negative results indicating LAB. Spoilage bacteria were enumerated using nutrient agar (NA) plates prepared in the same manner and incubated under aerobic conditions and anaerobic conditions at 37 °C for 48 h. The in situ antibacterial activity of clove and thyme EOs was assessed by comparing bacterial counts across treatments, controls, and Amoxicillin-supplemented samples, allowing for direct evaluation of the antimicrobial potential of the EOs.

Statistical Analysis. Data were analyzed using one-way analysis of variance (ANOVA) and independent t tests to evaluate the effects of treatments on measured variables. ANOVA was used to assess overall differences among treatment groups, and Tukey's Honest Significant Difference (HSD) test was applied for posthoc pairwise comparisons to identify specific differences at a 95% confidence level ($p < 0.05$). Additionally, independent t tests were used to directly compare specific treatments where appropriate. Means for each group were calculated, and significant differences were reported. Statistical analyses were conducted using Python (version 3.8) with appropriate statistical libraries, and data visualization was performed using heatmaps and standard plotting tools.

RESULTS AND DISCUSSION

Molecular Identification of Chemical Constituents via GC-MS. The chemical compositions of clove oil and thyme oil were analyzed using GC-MS, revealing distinct profiles with unique dominant and minor compounds in each oil. Clove oil is primarily defined by phenolic compounds, with eugenol as the major component, while thyme oil exhibits a broader array of monoterpenes and terpenoids. These differences underline their potential applications in diverse fields such as food preservation, pharmaceuticals, and cosmetics (Table 2 and Figure 1).

Table 2. Chemical Composition of Clove and Thyme Oils by Means of GC-MS

		Area %	
Compound	RT	Clove Oil	Thyme Oil
α-Pinene	5.100	N.D.	18.00
Camphene	5.200	N.D.	3.50
β-Pinene	5.431	N.D.	0.57
p-Menthane Z	5.516	N.D.	0.71
β-Myrcene	5.770	N.D.	2.49
Cyclofenchene	5.993	N.D.	0.32
Isocineole	6.190	N.D.	0.19
β-Cymene	6.536	N.D.	26.73
D-Limonene	6.665	N.D.	6.19
γ-Terpinene	7.183	N.D.	1.74
α,p-Dimethylstyrene	7.704	N.D.	0.25
(+)-4-Carene	7.833	N.D.	1.75
β-Linalool	8.078	N.D.	2.77
Fenchol	8.286	N.D.	0.57
β-Terpineol	8.928	N.D.	1.13
(−)-Borneol	9.411	N.D.	1.67
α-Terpineol	10.202	N.D.	4.68
Thymol	12.565	N.D.	27.53
Eugenol	14.217	73.45	N.D.
(+)-Longifolene	14.879	N.D.	0.69
β-Caryophyllene	15.047	9.00	N.D.
β-Ylangene	15.131	0.24	N.D.
β-Bisabolene	15.494	1.18	N.D.
Carvacrol	15.500	N.D.	1.50
Eugenyl Acetate	16.194	11.95	N.D.
Isoeugenol	16.244	1.64	N.D.
Alloaromadendrene	17.020	0.27	N.D.
Spathulenol	17.096	0.89	N.D.
Caryophyllene oxide	18.159	N.D.	0.11

In clove oil, eugenol emerged as the dominant compound, accounting for 73.45% of the total area. This was followed by eugenyl acetate (11.95%), which enhances the oil's overall chemical efficacy, and β-caryophyllene (9.00%), a sesquiterpene known for its anti-inflammatory properties. Trace amounts of β-ylangene (0.24%), β-bisabolene (1.18%), isoeugenol (1.64%), alloaromadendrene (0.27%), and spathulenol (0.89%) were also detected, contributing minimally to the overall composition but adding complexity to the oil's profile.

Thyme oil, in contrast, showed a more diverse composition. β-Cymene was the most abundant compound, comprising 26.73% of the total area, followed by thymol (27.53%) and α-pinene (18.00%). Other significant constituents included D-limonene (6.19%), camphene (3.50%), and carvacrol (1.50%).

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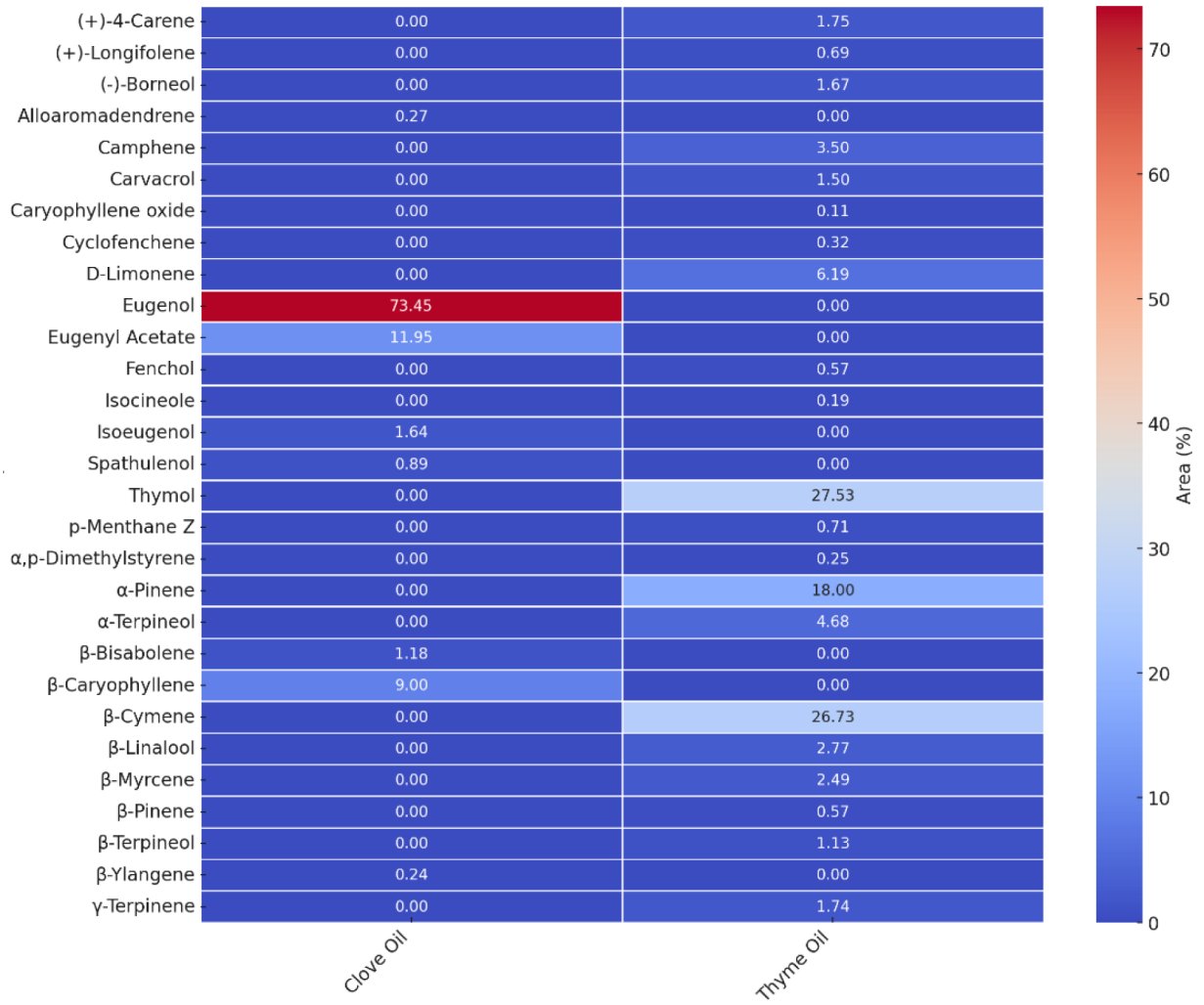

Figure 1. Heatmap illustrating the relative abundance (Area %) of key compounds in clove and thyme oils based on GC-MS analysis.

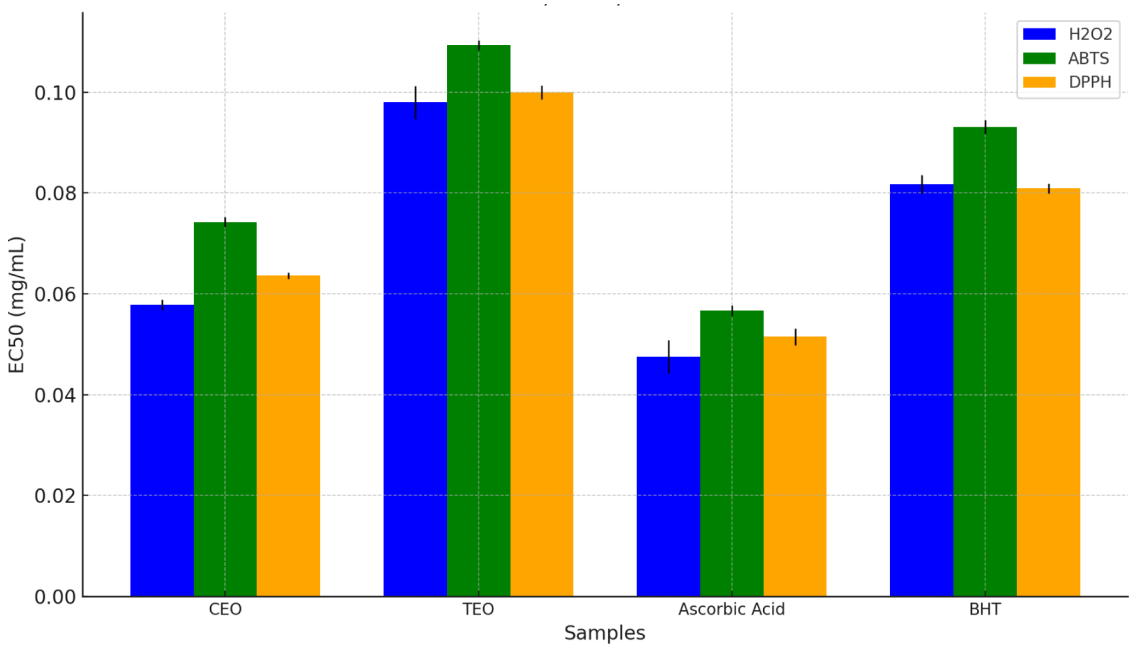

Figure 2. EC_{50} values of clove and thyme essential oils compared to ascorbic acid and BHT in H_2O_2, DPPH, and ABTS assays. The values were calculated based on the average values of the scavenging activity percentages obtained from three independent samples.

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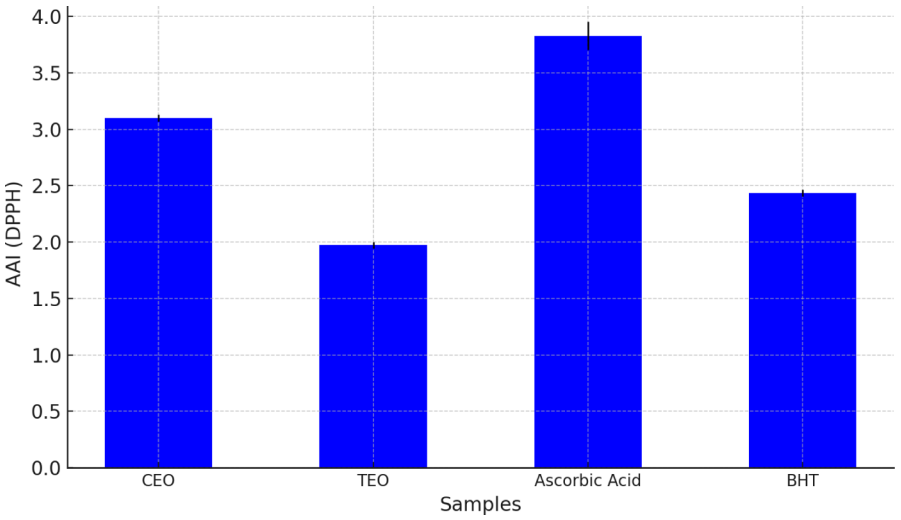

Figure 3. Antioxidant activity index of clove and thyme oils compared to standard antioxidants (ascorbic acid and BHT) against DPPH.

Minor components such as (+)-4-carene (1.75%), γ-terpinene (1.74%), and β-linalool (2.77%) were also detected, contributing to the oil's complexity.

The reported data in our study align with previous findings on the chemical composition of thyme and clove essential oils. Thymol and carvacrol are consistently identified as the major components of thyme essential oil, with significant contributions from *p*-cymene and γ-terpinene.[8,12,32] These compounds are key to TEO's antimicrobial and antioxidant properties.

For clove essential oil, eugenol remains the predominant compound, comprising 70−90% of its content. However, other notable constituents include eugenyl acetate and β-caryophyllene, which rank second and third in concentration, respectively. Eugenyl acetate contributes significantly to the oil's aroma and bioactivity, while β-caryophyllene, a sesquiterpene, is recognized for its anti-inflammatory and antifungal properties.[8,9,33] Minor constituents such as α-humulene and methyl salicylate have also been reported, contributing to the oil's functional properties.[9]

Antioxidant Activity. Antioxidant activity of clove oil and thyme oil was evaluated utilizing three well-known methods: H_2O_2 scavenging, DPPH radical scavenging, and ABTS radical cation scavenging assays (Figure 2). These tests were carried out to compare the effectiveness of the oils with standard antioxidants, ascorbic acid and BHT, and to explore their potential as natural alternatives to synthetic compounds.

In the H_2O_2 scavenging test, ascorbic acid stood out as the most effective antioxidant, requiring the lowest concentration (EC_{50} = 0.049 mg/mL) to neutralize half of the free radicals. Clove oil followed closely, with an EC50 of 0.058 mg/mL, demonstrating strong activity. On the other hand, thyme oil required a higher concentration (EC_{50} = 0.102 mg/mL) to achieve the same effect, indicating relatively weaker activity, while BHT performed moderately (EC_{50} = 0.081 mg/mL). The DPPH radical scavenging test showed a similar trend. Ascorbic acid again demonstrated the highest potency (EC_{50} = 0.052 mg/mL), while clove oil (EC_{50} = 0.063 mg/mL) showed notable activity, even surpassing BHT (EC_{50} = 0.081 mg/mL). Thyme oil, with an EC_{50} of 0.106 mg/mL, displayed the weakest performance in this assay. The ABTS test further confirmed these patterns, with ascorbic acid once more leading the way (EC_{50} = 0.057 mg/mL), followed by clove oil (0.074 mg/mL). Thyme oil, however, lagged behind with the highest EC_{50} (0.124 mg/mL), indicating lower antioxidant potential.

To provide a standardized comparison, the antioxidant activity index (AAI) was calculated based on the EC_{50} results (Figure 3). Ascorbic acid had the highest AAI value (3.79), classifying it as a very strong antioxidant. Notably, clove oil achieved a similarly strong result, with an AAI of 3.12, highlighting its potential as a natural antioxidant. BHT, a commonly used synthetic antioxidant, recorded an AAI of 2.44, placing it in the strong category. Thyme oil, with an AAI of 1.85, exhibited moderate-to-strong antioxidant activity.

The antioxidant activity of both clove and thyme essential oils has been extensively documented, with each exhibiting distinct mechanisms of action, largely driven by their chemical compositions.[11,12,32,34] The strong antioxidant activity of clove essential oil can be primarily attributed to its high concentration of eugenol (approximately 75%), a phenolic compound known for its potent radical-scavenging properties.[35,36] Eugenol operates by donating hydrogen atoms from its hydroxyl group to neutralize reactive oxygen species (ROS), thereby preventing oxidative damage to biological molecules.[35,36] This mechanism is particularly effective in assays like DPPH and ABTS, where eugenol's capacity to stabilize free radicals is evident, reflected in its low EC_{50} values.[11] Moreover, eugenol inhibits lipid peroxidation, thereby protecting cellular membranes and other lipid structures from oxidative degradation. The presence of eugenyl acetate and β-caryophyllene in clove oil likely complements these effects by enhancing the oil's stability and providing additional antioxidant efficacy through synergistic interactions.[35,36]

In contrast, thyme essential oil, which exhibited weaker activity across all antioxidant assays, contains a diverse mix of monoterpenes and terpenoids, including β-cymene, thymol, and α-pinene. In this context, Chroho et al. (2024)[37] explored the antioxidant properties of *T. zygis* essential oil (TZEO), focusing on its key compounds, carvacrol and thymol. The DPPH test revealed that TZEO had an IC_{50} value of 3.27 ± 0.16 mg/mL, showing its ability to reduce free radicals, although it was less effective than *O. compactum* essential oil and ascorbic acid. The antioxidant activity of TZEO was strongly linked to the presence of carvacrol and thymol, both known for their powerful free radical scavenging properties. These compounds, with their hydroxyl, methyl, and isopropyl

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groups, effectively neutralize free radicals by donating hydrogen atoms or electrons. Further, the FRAP test, which evaluates the ability to reduce ferric ions, showed that TZEO had an EC_{50} value of 2.16 ± 0.13 mg/mL, again demonstrating its antioxidant capacity, though it was weaker compared to ascorbic acid. Carvacrol and thymol play a crucial role in this activity due to their chemical structures, which allow them to stabilize and neutralize free radicals and ferric ions. This study highlights the contribution of these two compounds to the overall antioxidant activity of TZEO, emphasizing their importance in the oil's ability to combat oxidative stress.

While TEO compounds possess some antioxidant potential, their efficacy is generally lower compared to the phenolic compounds in CEO, particularly eugenol.[11] The lower concentration of individual active components in TEO may also dilute its overall antioxidant potential. Zengin and Baysal[11] reported that thyme oil showed weaker DPPH and FRAP values compared to clove oil, consistent with the hypothesis that phenolic compounds, especially eugenol, are more effective in scavenging free radicals than the compounds found in thyme oil. This observation was also reflected in studies where clove oil showed higher FRAP values and better performance in lipid oxidation inhibition, further supporting its superior antioxidant capacity.

In Vitro Evaluation of Antibacterial Activity. The antibacterial activity of clove essential oil and thyme essential oil was assessed using inhibition zones (Table 3 and Figure 4), minimum inhibitory concentration (MIC; Figure 5), and the selectivity index (SI; Figure 6) to determine their effectiveness and specificity against pathogens and LAB strains.

Inhibition Zone Analysis. The antibacterial activity of CEO and TEO was evaluated across a range of concentrations (10, 25, 50, and 100 mg/L) against various bacterial strains. The results revealed a concentration-dependent effect, with significant differences between the two oils at higher concentrations.

At the lowest tested concentration (10 mg/L), neither CEO nor TEO produced measurable inhibition zones for any bacterial strain, indicating no antibacterial activity at this concentration.

At 25 mg/L, CEO demonstrated moderate antibacterial activity, with inhibition zones ranging from 11.0 ± 0.0 mm for *Escherichia coli* (EC22) to 15.0 ± 0.0 mm for *Staphylococcus aureus* (SA13). TEO exhibited significantly lower activity ($p < 0.05$) for several strains, such as *Lactobacillus brevis* (LB81) and *Staphylococcus epidermidis* (SE76), where inhibition zones were 0.0 mm and 9.0 ± 0.0 mm, respectively. For *Lactobacillus plantarum* (LM44), CEO produced a zone of 12.0 ± 0.0 mm, while TEO showed no inhibition (0.0 mm), indicating a significant difference ($p < 0.05$).

At 50 mg/L, CEO displayed strong antibacterial activity, with inhibition zones ranging from 12.5 ± 0.71 mm for *Pseudomonas aeruginosa* (PA53) to 19.0 ± 0.0 mm for *Staphylococcus aureus* (SA13). In contrast, TEO demonstrated reduced efficacy, with inhibition zones ranging from 11.0 ± 0.0 mm for *Escherichia coli* (EC22) to 16.0 ± 0.0 mm for *Streptococcus pyogenes* (SS05). The differences between CEO and TEO were statistically significant ($p < 0.05$) for several strains, including *Staphylococcus aureus* (SA13) and *Pseudomonas aeruginosa* (PA53). For *Lactobacillus plantarum* (ST17), CEO produced an inhibition zone of 11.5 ± 0.71 mm, which was slightly larger than the 11.0 ± 0.0 mm observed with TEO.

Table 3. Sensitivity of Reference Bacteria to Clove and Thyme Essential Oils Assessed by Well Diffusion Assay (Inhibition Zone in mm)[a]

		Lactic acid bacteria (LAB)			Gram positive pathogenic bacteria			Gram negative pathogenic bacteria		
Concentration (mg/L)	Oil	ST17	LL55	LB81	LM44	SA13	SS05	EC22	PA53	SE76
10	CEO	–	–	–	–	–	–	–	–	–
	TEO	–	–	–	–	–	–	–	–	–
25	CEO	–	–	–	12.00 ± 0.00 a	15.00 ± 0.00 a	12.00 ± 0.00 a	11.00 ± 0.00 a	11.50 ± 0.71 a	13.00 ± 0.00 a
	TEO	–	–	–	–	14.00 ± 0.00 b	11.00 ± 0.00 b	12.00 ± 0.00 a	10.00 ± 0.00 b	9.00 ± 0.00 b
50	CEO	11.50 ± 0.71 a	–	12.00 ± 0.00 a	14.00 ± 1.41 a	19.00 ± 0.00 a	13.50 ± 0.71 a	15.00 ± 0.00 a	15.00 ± 0.00 a	12.50 ± 0.71 a
	TEO	11.00 ± 0.00 b	–	–	14.00 ± 0.00 a	14.00 ± 0.00 b	16.00 ± 0.00 b	13.00 ± 0.00 b	12.00 ± 0.00 b	11.00 ± 0.00 b
100	CEO	15.00 ± 1.41 a	19.00 ± 0.00 a	18.32 ± 1.66 a	16.00 ± 1.41 a	22.50 ± 4.95 a	14.50 ± 0.71 a	21.00 ± 0.00 a	21.00 ± 1.41 a	14.00 ± 0.00 a
	TEO	14.00 ± 0.00 a	14.00 ± 0.00 b	12.00 ± 0.00 b	15.00 ± 0.00 a	20.00 ± 0.00 a	18.50 ± 0.00 b	15.00 ± 0.00 b	14.00 ± 0.00 b	15.00 ± 0.00 a

[a]– refer to no inhibition.

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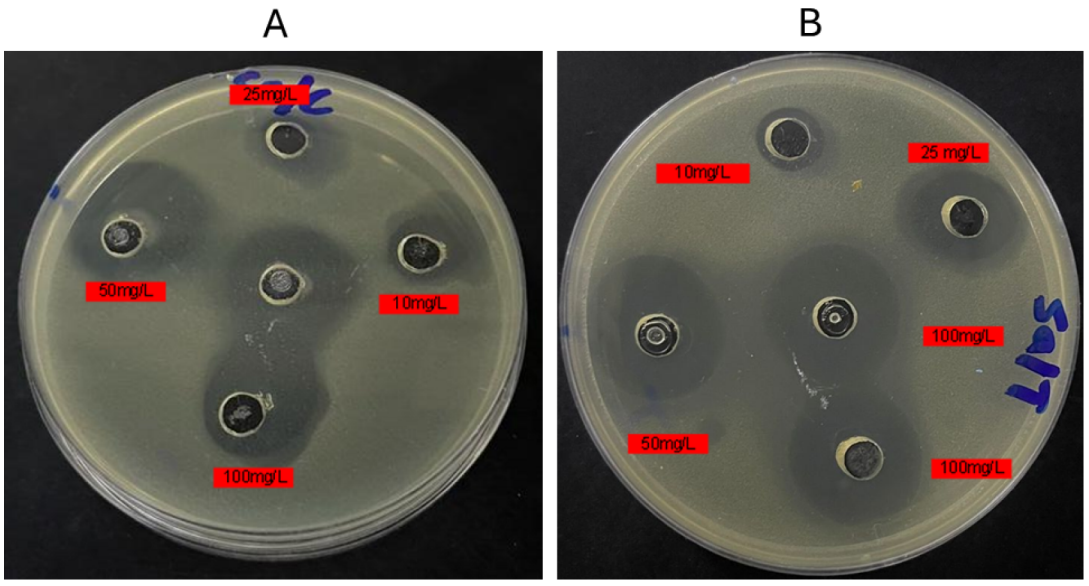

Figure 4. Zones of inhibition caused by different concentration of clove oil against *Staphylococcus aureus* ATCC 29213 (A) and thyme oil against *Staphylococcus aureus* ATCC 29213 using well diffusion assay (B).

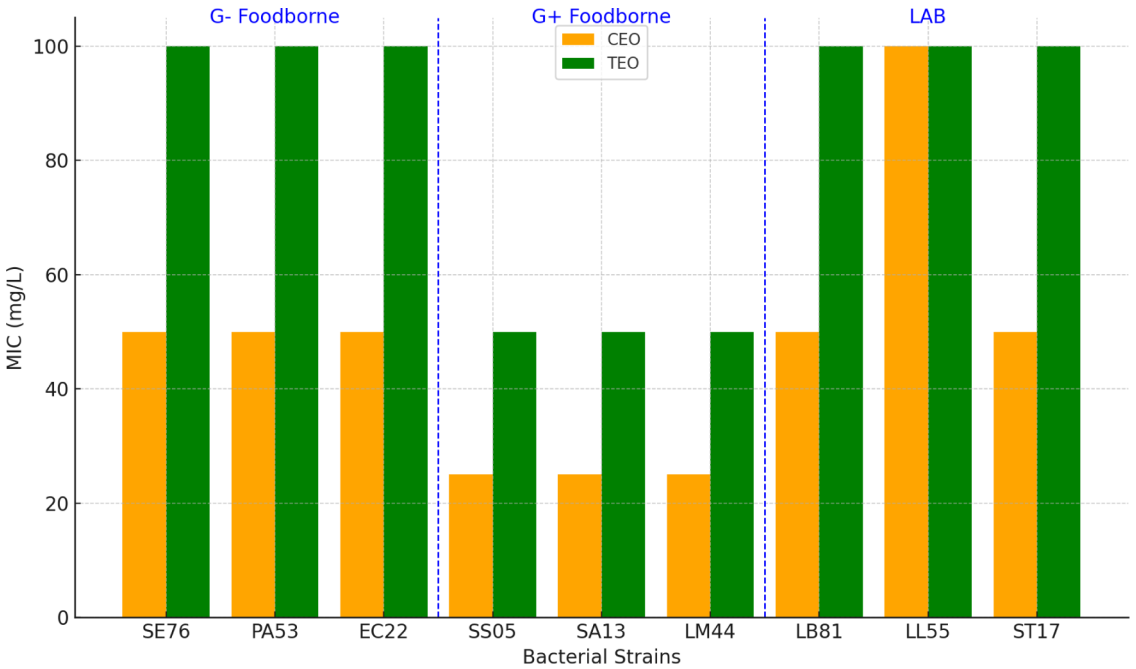

Figure 5. Minimum inhibitory concentration (MIC) of clove and thyme essential oils against LAB and foodborne pathogens. MIC values were calculated based on the average inhibition zones for each treatment.

At the highest tested concentration (100 mg/L), CEO demonstrated the largest inhibition zones, ranging from 14.0 ± 0.0 mm for *Staphylococcus epidermidis* (SE76) to 22.5 ± 4.95 mm for *Staphylococcus aureus* (SA13). TEO also exhibited antibacterial activity at this concentration, with inhibition zones ranging from 12.0 ± 0.0 mm for *Lactobacillus brevis* (LB81) to 20.0 ± 0.0 mm for *Staphylococcus aureus* (SA13). Statistically significant differences ($p < 0.05$) were observed for strains such as *Escherichia coli* (EC22), where CEO produced a zone of 21.0 ± 0.0 mm compared to 15.0 ± 0.0 mm for TEO, and *Lactobacillus brevis* (LB81), where CEO produced a zone of 18.32 ± 1.66 mm compared to 12.0 ± 0.0 mm for TEO. For *Lactobacillus plantarum* (ST17), CEO produced a zone of 15.0 ± 1.41 mm, slightly larger than the 14.0 ± 0.0 mm zone observed with TEO.

The MIC values further highlighted the superior antibacterial activity of CEO compared to TEO (Figure 5). For Gram-negative pathogens, CEO showed lower MIC values, with *Escherichia coli* (EC22) and *Pseudomonas aeruginosa* (PA53) both requiring 50 mg/L for inhibition, compared to 100 mg/L for TEO. Similarly, for Gram-positive pathogens, CEO exhibited lower MIC values, with *Staphylococcus aureus* (SA13) and *Staphylococcus saprophyticus* (SS05) requiring 25 mg/L compared to 50 mg/L for TEO. LAB strains were less sensitive overall, with MIC values ranging from 50 to 100 mg/L for CEO and consistently 100 mg/L for TEO.

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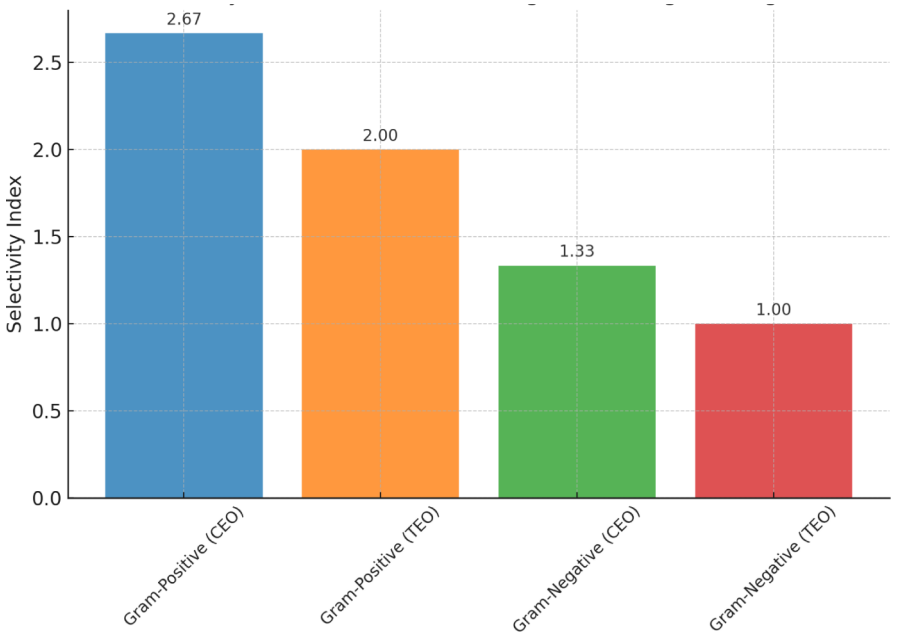

Figure 6. Selectivity index of clove and thyme essential oils for pathogenic bacteria over beneficial LAB strains.

The antimicrobial activity of TEO and CEO can be attributed to their major bioactive components, which disrupt bacterial cell integrity and function.[8,9,11,12] TEO contains key components such as thymol, carvacrol, β-cymene, and linalool, which disrupt bacterial cell membranes, increasing permeability and causing leakage of essential intracellular contents, resulting in cell death.[38,39] β-Cymene enhances the membrane-disrupting activities of thymol and carvacrol, leading to effective inhibition of Gram-positive bacteria like *Staphylococcus aureus*.[40] However, its efficacy is reduced against Gram-negative bacteria due to their protective outer membranes.[8,12,13]

CEO's antimicrobial action is primarily due to its high eugenol content. Eugenol's lipophilic nature enables it to penetrate microbial cell membranes, disrupting structural integrity and causing leakage of intracellular contents.[9,41,42] Additionally, eugenol inhibits key enzymatic processes critical for bacterial survival.[10,13] CEO effectively inhibits both Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhi*.[9] Its strong antifungal properties are also attributed to synergistic actions with eugenyl acetate and β-caryophyllene. Another key mechanism involves the inhibition of quorum sensing, the bacterial communication system that regulates virulence factors and biofilm formation.[43] By disrupting quorum sensing, CEO and TEO reduce bacterial virulence and biofilm resilience, making pathogens more vulnerable to antimicrobial agents and environmental stressors.[43] These mechanisms highlight the potential of TEO and CEO as natural alternatives to synthetic antimicrobials. While TEO demonstrates stronger activity against Gram-positive bacteria, CEO's broader spectrum of action makes it particularly effective across a wide range of pathogens.

In this context, Chroho et al. (2024)[37] demonstrated the antimicrobial activity of *Thymus zygis*essential oil (TZEO), primarily due to its main components, thymol and carvacrol. These phenolic compounds disrupt bacterial membranes, collapsing the proton motive force, depleting ATP, and causing cell death. TZEO showed strong activity against Gram-positive bacteria such as *Staphylococcus aureus* and *Listeria monocytogenes*, which are more vulnerable to hydrophobic compounds due to their simpler membrane structure. Minor compounds like *p*-cymene and borneol enhance TZEO's efficacy. *P*-cymene aids thymol and carvacrol penetration by integrating into bacterial membranes, while borneol's water solubility allows efficient membrane penetration. Though Gram-negative bacteria such as *Escherichia coli* and *Salmonella enterica* are generally more resistant due to complex outer membranes, TZEO still demonstrated activity, as thymol and carvacrol interact with lipopolysaccharides and proteins.

Selectivity Index (SI). Selectivity Index (SI) was calculated to evaluate preferential activity of clove essential oil and thyme essential oil against Gram-positive and Gram-negative pathogens compared to LAB strains (Figure 6). For Gram-positive pathogens, CEO demonstrated an SI of 2.67, indicating its strong selectivity for pathogens over LAB. TEO exhibited a slightly lower SI of 2.0 for Gram-positive bacteria, reflecting its broad-spectrum antimicrobial activity but reduced selectivity compared to CEO.

For Gram-negative pathogens, the SI values were lower for both oils, with CEO showing an SI of 1.33 and TEO showing an SI of 1.0. This suggests that both oils are less selective against Gram-negative bacteria, likely due to the protective outer membrane of these pathogens, which reduces the oils' relative efficacy.

Overall, CEO displayed higher selectivity for both Gram-positive and Gram-negative pathogens over LAB strains compared to TEO.

Combining the inhibition zone, MIC, and SI results highlights the superior antimicrobial properties of CEO across both Gram-positive and Gram-negative pathogens. CEO consistently exhibited larger inhibition zones, lower MIC values, and a higher SI, making it a more potent and selective antimicrobial agent compared to TEO. These findings suggest that CEO holds greater potential for applications requiring broad-spectrum and selective antibacterial activity.

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In this context, previous studies indicated that essential oils could perform a selective antimicrobial effects against pathogenic bacterial.[44,45] Citrus oils have been shown to possess a high level of selectivity against pathogenic bacteria, including *Escherichia coli* and *Salmonella Enteritidis*, while showing minimal inhibitory effects on beneficial bacteria such as *Lactobacillus plantarum* and *Lactobacillus rhamnosus*.

Molecular Docking Analysis of CEO and TEO Compounds. To elucidate the selectivity of the examined oils, molecular docking was utilized based on the selectivity index. This analysis focused on the interactions of β-cymene, eugenol, and thymol—key components of clove and thyme oils—with essential bacterial enzymes. The study included both pathogenic strains (*Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*) and a beneficial strain (*Lactiplantibacillus plantarum*).

The binding affinities of the essential oil compounds varied depending on the enzyme and bacterial strain, with higher affinities generally observed for pathogenic bacterial enzymes (Table 4 and Figures 7−11).

One of the enzymes examined was catalase, which helps bacteria survive oxidative stress by breaking down hydrogen peroxide. Eugenol and thymol were found to strongly inhibit catalase in *E. coli* and *S. aureus* (binding energy: −6.5 kcal/mol), making these bacteria more vulnerable to oxidative conditions. On the other hand, their interaction with catalase in LAB was much weaker, allowing *L. plantarum* to maintain its natural defenses against oxidative stress, which is essential for its survival in environments like fermented foods.

Another enzyme, esterase, plays a role in lipid metabolism and maintaining the integrity of bacterial membranes. In pathogenic bacteria like *L. monocytogenes*, thymol and eugenol strongly inhibited esterase (−5.9 and −5.8 kcal/mol, respectively), which could disrupt membrane stability and reduce the bacteria's ability to adapt. In LAB, however, the binding to esterase was minimal, ensuring minimal disruption to their metabolic functions.

Phosphatases are involved in phosphate metabolism and cellular signaling, crucial for maintaining cellular homeostasis across bacterial species. These enzymes facilitate essential biochemical pathways, including nutrient acquisition and cell wall remodeling. In pathogenic bacteria, phosphatases showed moderate affinity for eugenol and thymol, potentially disrupting phosphate metabolism and cellular signaling. However, LAB-specific alkaline phosphatase exhibited lower binding affinities (−6.1 kcal/mol for eugenol and −5.6 kcal/mol for thymol), preserving its role in nutrient acquisition and cell wall remodeling.

Proteases are responsible for protein turnover and cellular repair, enabling bacteria to adapt to environmental stressors by recycling amino acids and maintaining structural proteins with binding energies of −5.2 kcal/mol for eugenol and −5.0 kcal/mol for thymol. Protease activity in pathogenic strains was notably inhibited by both CEO and TEO compounds, interfering with protein turnover and cellular repair mechanisms. This inhibition compromises bacterial adaptation to environmental stress. In LAB, protease interactions with CEO and TEO compounds were weaker, preserving structural protein maintenance and supporting fermentation processes.

Finally, amylase supports carbohydrate breakdown, ensuring efficient energy utilization during fermentation. LAB-specific amylase showed negligible binding interactions with CEO and TEO compounds, ensuring uninterrupted carbohydrate breakdown and energy generation during fermentation.

Practically, the molecular docking results show that CEO and TEO compounds selectively inhibit key enzymes in harmful bacteria while preserving the activity of the same enzymes in beneficial bacteria. This aligns with the results of *in vitro* antimicrobial tests, where both oils demonstrated selective activity against pathogens without negatively affecting LAB.

In this context, a molecular docking study was conducted to explore the binding interactions between eugenol and enoyl-[acyl-carrier-protein] reductase [NADPH] FabI enzyme from *Staphylococcus aureus*.[10] The results showed that eugenol could bind to FabI—a crucial enzyme involved in bacterial fatty acid biosynthesis—via multiple interactions with key amino acids such as Thr145, Lys164, Tyr157, and Tyr147, forming stable complexes. This interaction suggested that the antibacterial effect of eugenol may be mediated through inhibition of fatty acid biosynthesis, leading to ROS accumulation and bacterial death. The DNA cleavage assay further confirmed that eugenol could induce DNA damage, inhibiting bacterial replication.

In another study, molecular docking was used to evaluate the interaction between thyme oil compounds and DNA polymerase, RNA polymerase, and topoisomerase II from *Staphylococcus aureus*, *Salmonella enterica Typhimurium*, and *Listeria monocytogenes*.[46] The results showed that the major components, particularly thymol and β-sesquiphellandrene, had strong binding interactions with the target bacterial enzymes. These interactions were characterized by hydrogen bonding, hydrophobic interactions, and van der Waals forces. Thymol exhibited the most potent binding to the target proteins, indicating its potential as a key antibacterial agent. The docking results suggested that these compounds could effectively inhibit the activity of bacterial enzymes involved in DNA replication and transcription, contributing to their observed antibacterial effects.

The inhibitory effects of essential oils are not limited to clove and thyme but extend to other oils with potent bioactive compounds. For instance, the study of Jianu et al. (2021)[47] investigated the antibacterial and antioxidant activities of compounds from from *Ruta graveolens* L. essential oil (RGEO) using molecular docking to identify potential targets and interactions. The study revealed a strong affinity of several compounds toward DDl (2I80), a crucial enzyme involved in bacterial wall biosynthesis, with binding energies exceeding that of the native ligand. Among these, 4-(3,4-Methylenedioxyphenyl)-2-butanone exhibited the highest binding affinity, forming multiple hydrogen bonds and hydrophobic interactions at the DDl binding site, effectively mimicking the interaction pattern of the native ligand. These findings align with prior research highlighting the bactericidal properties of monoterpenes, which disrupt bacterial walls by interfering with their structural integrity.

In addition to antibacterial activity, RGEO compounds demonstrated significant antioxidant potential through interactions with key antioxidant-related proteins. Docking studies identified xanthine oxidase (XO) and lipoxygenase (LOX) as primary targets. 4-(3,4-Methylenedioxyphenyl)-2-butanone, the highest-affinity ligand for XO, formed stabilizing interactions comparable to the native ligand hypoxanthine, underscoring its potential role in mitigating oxidative stress and lowering uric acid levels. Furthermore, 4-Carene exhibited the strongest inhibitory effect on LOX, forming multiple

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Table 4. Binding Affinity and Mechanisms of Three CEO and TEO Compounds against Pathogenic and LAB Bacteria Enzymes

Bacteria	Compound	Enzyme	Potential Binding Mechanism	Functional Groups Involved	Binding Energy (kcal/mol)	Amino Acid Residues	Active Site Binding
Escherichia coli	β-Cymene	Catalase	Hydrophobic interaction	Alkyl groups (C−H), Phenyl ring (C−H)	−6.4	N201, F214, R125, R165, R411, S414	Yes
Escherichia coli	β-Cymene	Esterase	Hydrophobic interaction	Alkyl groups (C−H), Phenyl ring (C−H)	−5.5	G233, H234, W235, H237, A238, E239	Yes
Escherichia coli	β-Cymene	Phosphatase	Hydrophobic interaction	Alkyl groups (C−H), Phenyl ring (C−H)	−4.9	D8, D10, T16, E17, W20, F24, F41	Yes
Escherichia coli	β-Cymene	Protease	None	Alkyl groups (C−H), Phenyl ring (C−H)	None	None	No
Escherichia coli	Eugenol	Catalase	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−6.3	N201, F214, R125, R165, R411, S414	Yes
Escherichia coli	Eugenol	Esterase	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−5.2	G233, H234, W235, H237, A238, E239	Yes
Escherichia coli	Eugenol	Phosphatase	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−5.1	D8, D10, T16, E17, W20, F24, F41	Yes
Escherichia coli	Eugenol	Protease	None	Hydroxyl group (OH), Phenyl ring (C−H)	None	None	No
Escherichia coli	Thymol	Catalase	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−5.8	H128, R165	Yes
Escherichia coli	Thymol	Esterase	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−5.2	G233, H234, W235, H237, A238, E239	Yes
Escherichia coli	Thymol	Phosphatase	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−5.2	D8, D10, T16, E17, W20, F24, F41	Yes
Escherichia coli	Thymol	Protease	None	Hydroxyl group (OH), Phenyl ring (C−H)	None	None	No
Listeria monocytogenes	β-Cymene	Catalase	Hydrophobic interaction	Alkyl groups (C−H), Phenyl ring (C−H)	−5.6	P350, I351	Yes
Listeria monocytogenes	β-Cymene	Esterase	Hydrophobic interaction	Alkyl groups (C−H), Phenyl ring (C−H)	−5.8	S30, K52, A58, M59, E60, W61, L64, S68	Yes
Listeria monocytogenes	β-Cymene	Phosphatase	Hydrophobic interaction	Alkyl groups (C−H), Phenyl ring (C−H)	−4.9	D8, D10, T16, E17, W20, F24, F41, C44	Yes
Listeria monocytogenes	β-Cymene	Protease	Hydrophobic interaction	Alkyl groups (C−H), Phenyl ring (C−H)	−5.5	Q204, L205, V244	Yes
Listeria monocytogenes	Eugenol	Catalase	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−5.7	N345, Y346, L347, Q348, K370, Q371	Yes
Listeria monocytogenes	Eugenol	Esterase	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−5.0	S30, K52, A58, M59, E60, W61, L64, S68	Yes
Listeria monocytogenes	Eugenol	Phosphatase	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−5.1	D8, D10, T16, E17, W20, F24, F41, C44	Yes
Listeria monocytogenes	Eugenol	Protease	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−5.1	L33, R34, P36, Y37, L65, D112, Q114, T115	Yes
Listeria monocytogenes	Thymol	Catalase	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−5.9	N345, Y346, L347, Q348, K370, Q371	Yes
Listeria monocytogenes	Thymol	Esterase	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−5.4	S30, K52, A58, M59, E60, W61, L64, S68	Yes
Listeria monocytogenes	Thymol	Phosphatase	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−5.2	D8, D10, T16, E17, W20, F24, F41, C44	Yes
Listeria monocytogenes	Thymol	Protease	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−5.5	Q204, L205, V244	Yes

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Table 4. continued

Bacteria	Compound	Enzyme	Potential Binding Mechanism	Functional Groups Involved	Binding Energy (kcal/mol)	Amino Acid Residues	Active Site Binding
Pseudomonas aeruginosa	β-Cymene	Catalase	Hydrophobic interaction	Alkyl groups (C−H), Phenyl ring (C−H)	−6.5	N128	Yes
Pseudomonas aeruginosa	β-Cymene	Esterase	Hydrophobic interaction	Alkyl groups (C−H), Phenyl ring (C−H)	−5.8	R12, S13, S14, L17, D60, V61, G64, W65	Yes
Pseudomonas aeruginosa	β-Cymene	Phosphatase	Hydrophobic interaction	Alkyl groups (C−H), Phenyl ring (C−H)	−4.9	D13, H8, E63, S65, H38, A71, T72, H74, R98	Yes
Pseudomonas aeruginosa	β-Cymene	Protease	Hydrophobic interaction	Alkyl groups (C−H), Phenyl ring (C−H)	−4.9	F162, R165, A200, D202, D208, D250, R266	Yes
Pseudomonas aeruginosa	Eugenol	Catalase	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−6.5	N128	Yes
Pseudomonas aeruginosa	Eugenol	Esterase	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−5.5	R12, S13, S14, L17, D60, V61, G64, W65	Yes
Pseudomonas aeruginosa	Eugenol	Phosphatase	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−5.5	D13, H8, E63, S65, H38, A71, T72, H74, R98	Yes
Pseudomonas aeruginosa	Eugenol	Protease	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−4.9	F162, R165, A200, D202, D208, D250, R266	Yes
Pseudomonas aeruginosa	Thymol	Catalase	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−6.5	H55, Y338	Yes
Pseudomonas aeruginosa	Thymol	Esterase	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−5.7	R12, S13, S14, L17, D60, V61, G64, W65	Yes
Pseudomonas aeruginosa	Thymol	Phosphatase	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−5.1	D13, H8, E63, S65, H38, A71, T72, H74, R98	Yes
Pseudomonas aeruginosa	Thymol	Protease	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−5.0	F162, R165, A200, D202, D208, D250, R266	Yes
Staphylococcus aureus	β-Cymene	Catalase	Hydrophobic interaction	Alkyl groups (C−H), Phenyl ring (C−H)	−6.0	N302, Y303, D306, V307	Yes
Staphylococcus aureus	β-Cymene	Esterase	Hydrophobic interaction	Alkyl groups (C−H), Phenyl ring (C−H)	−5.7	E198, Q199, D200, A201, V202, V276, F279	Yes
Staphylococcus aureus	β-Cymene	Phosphatase	Hydrophobic interaction	Alkyl groups (C−H), Phenyl ring (C−H)	−5.0	K140, H164, P180, V181, A177, P178	Yes
Staphylococcus aureus	β-Cymene	Protease	Hydrophobic interaction	Alkyl groups (C−H), Phenyl ring (C−H)	−5.3	F42, R51, T79, N81, T131, Q132, R156	Yes
Staphylococcus aureus	Eugenol	Catalase	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−6.5	N302, Y303, D306, V307	Yes
Staphylococcus aureus	Eugenol	Esterase	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−5.6	D184, A185, L187, I192, L196, S204, Q205	Yes
Staphylococcus aureus	Eugenol	Phosphatase	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−5.0	K140, H164, P180, V181, A177, P178	Yes
Staphylococcus aureus	Eugenol	Protease	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−5.1	F42, R51, T79, N81, T131, Q132, R156	Yes
Staphylococcus aureus	Thymol	Catalase	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−5.7	N302, Y303, D306, V307	Yes
Staphylococcus aureus	Thymol	Esterase	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−5.9	D184, A185, L187, I192, L196, S204, Q205	Yes
Staphylococcus aureus	Thymol	Phosphatase	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−4.9	K140, H164, P180, V181, A177, P178	Yes
Staphylococcus aureus	Thymol	Protease	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−5.3	F42, R51, T79, N81, T131, Q132, R156	Yes

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Table 4. continued

Bacteria	Compound	Enzyme	Potential Binding Mechanism	Functional Groups Involved	Binding Energy (kcal/mol)	Amino Acid Residues	Active Site Binding
Lactiplantibacillus plantarum	β-Cymene	Alkaline Phosphatase	Hydrophobic interaction	Alkyl groups (C−H), Phenyl ring (C−H)	−5,3	H74,Q75, V76, G77, A285, L288, Q289, F348, R349	Yes
Lactiplantibacillus plantarum	β-Cymene	Amylase	None	Alkyl groups (C−H), Phenyl ring (C−H)	None	L467,W517,W520,M526,T552,V553,V554,V555,L580,I584,Q585,L588,R599	No
Lactiplantibacillus plantarum	β-Cymene	Protease	Hydrophobic interaction	Alkyl groups (C−H), Phenyl ring (C−H)	−5,1	D14,Y18,R19,D20,T25,F221	No
Lactiplantibacillus plantarum	Eugenol	Alkaline Phosphatase	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−6,1	H47,Q75, V76, G77, A285, L288, Q289, F348, R349	Yes
Lactiplantibacillus plantarum	Eugenol	Amylase	None	Hydroxyl group (OH), Phenyl ring (C−H)	None	L467,W517,W520,M526,T552,V553,V554,V555,L580,I584,Q585,L588,R599	No
Lactiplantibacillus plantarum	Eugenol	Protease	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−4,4	H215,V219,L223,A224,F226,K228	No
Lactiplantibacillus plantarum	Thymol	Alkaline Phosphatase	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−5,6	Y259,L261,S262,S285,L288	Yes
Lactiplantibacillus plantarum	Thymol	Amylase	Hydrogen bonding	Hydroxyl group (OH), Phenyl ring (C−H)	−5,7	L467,W517,W520,M526,T552,V553,V554,V555,L580,I584,Q585,L588,R599	No
Lactiplantibacillus plantarum	Thymol	Protease	None	Hydroxyl group (OH), Phenyl ring (C−H)	None	D14,Y18,R19,D20,T25,F221	No

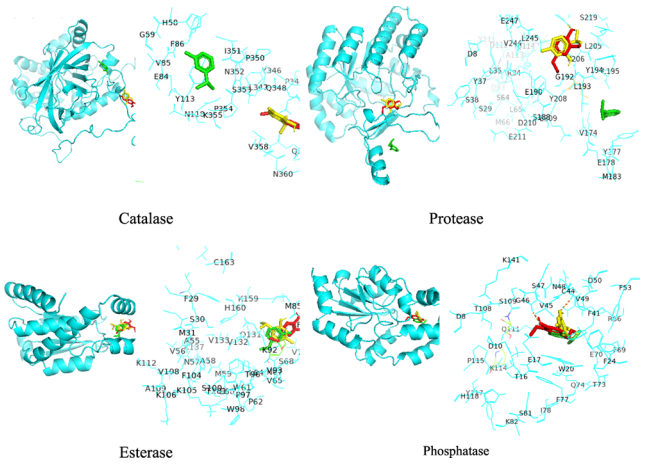

Figure 7. Molecular docking visualization of *Listeria monocytogenes* enzymes binding to key compounds from clove and thyme essential oils. The docking interactions are represented by different colors: eugenol (red), β-cymene (green), and thymol (yellow).

hydrophobic interactions that align with previous findings linking terpene-rich extracts to LOX inhibition. It is important to highlight that 4-carene is among the compounds that were reported in our sample.

Sensory Evaluation of Buttermilk Fortified with Essential Oils. The sensory evaluation of buttermilk samples treated with clove essential oil and thyme essential oil at concentrations of 50 and 100 μg per 100 mL milk was assessed for color, smell, taste, and overall acceptability (Figure 12). Statistical analysis revealed significant differences among treatments for specific attributes, along with clear trends within the samples.

For color, the control group achieved the highest average score (8.5), reflecting its superior visual appeal. TEO-treated samples maintained relatively high scores, with TEO at 100 μg scoring 7.57 and TEO at 50 μg achieving 7.29. CEO treatments scored lower, with CEO at 100 μg scoring 7.43 and CEO at 50 μg receiving the lowest score (7.14). These differences were not statistically significant (p = 0.149), suggesting minimal impact of essential oil treatments on color perception.

The smell attribute showed significant differences among treatments (p = 0.039). TEO at 100 μg achieved the highest average score (7.86), followed closely by TEO at 50 μg (7.64) and the control group (7.57). CEO treatments lagged behind, with CEO at 100 μg scoring 7.21 and CEO at 50 μg receiving the lowest score (6.93). These findings highlight the ability of thyme oil, particularly at higher concentrations, to enhance the aromatic profile of buttermilk, whereas clove oil had a more subdued impact on smell.

Taste scores revealed statistically significant differences among treatments (p < 0.0001). TEO at 100 μg outperformed all other treatments, achieving the highest average score (7.93). TEO at 50 μg and the control group both scored 7.14, indicating comparable flavor profiles. CEO treatments performed poorly, with CEO at 100 μg scoring 6.14 and CEO at 50 μg receiving the lowest score (5.86). These results demonstrate the superior ability of thyme essential oil to enhance taste, particularly at higher concentrations, while clove oil, especially at 50 μg, negatively impacted taste perception.

Overall acceptability scores mirrored the trends observed for taste. TEO at 100 μg scored the highest (7.86), slightly

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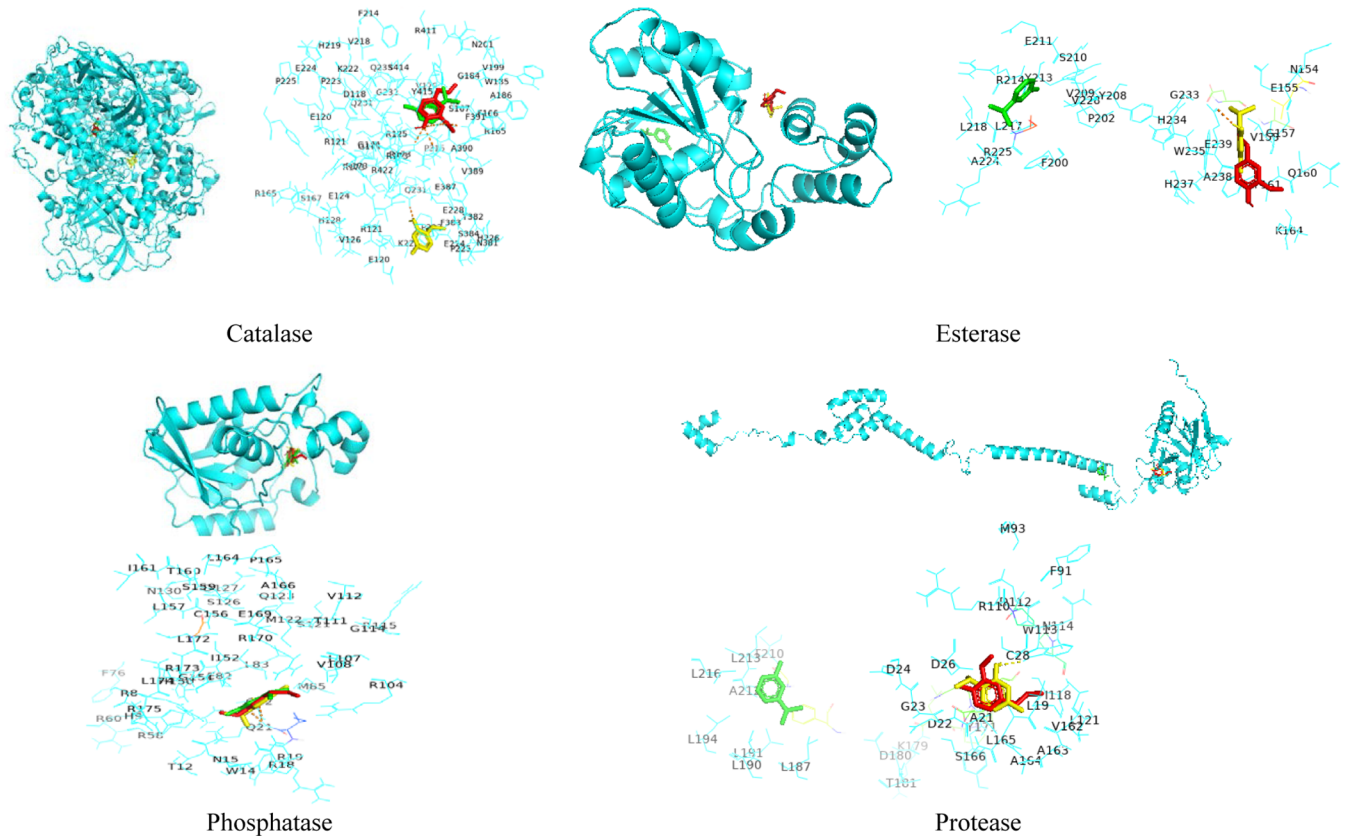

Figure 8. Molecular docking visualization of *E-coli* enzymes binding to key compounds from clove and thyme essential oils. The docking interactions are represented by different colors: eugenol (red), *β*-cymene (green), and thymol (yellow).

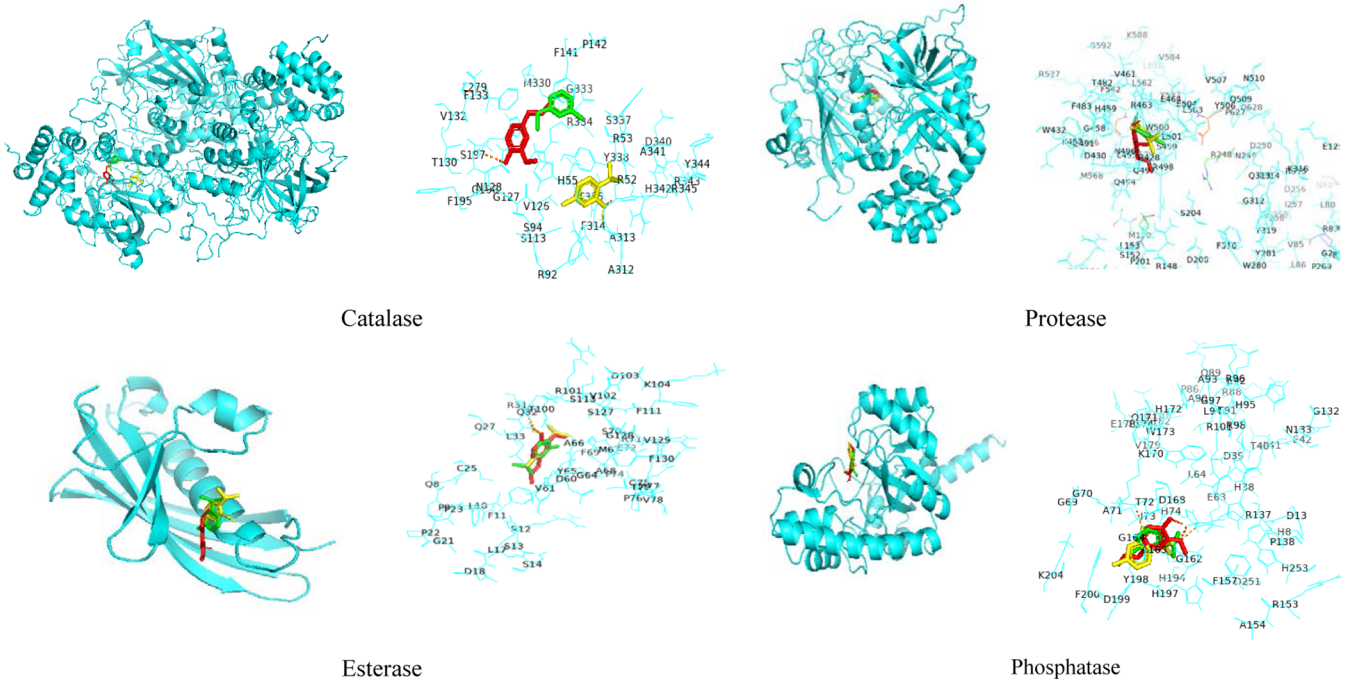

Figure 9. Molecular docking visualization of *Pseudomonas aeruginosa* enzymes binding to key compounds from clove and thyme essential oils. The docking interactions are represented by different colors: eugenol (red), *β*-cymene (green), and thymol (yellow).

surpassing the control group (7.79). TEO at 50 μg performed moderately well (7.07), while CEO treatments scored significantly lower, with CEO at 100 μg achieving 6.07 and CEO at 50 μg receiving the lowest score (5.64).

In this context, the study of Hamed et al.[18] evaluated whether fortifying yogurt with eucalyptus and myrrh essential oils affected sensory attributes compared to control. The results showed that yogurt fortified with 0.3% eucalyptus oil

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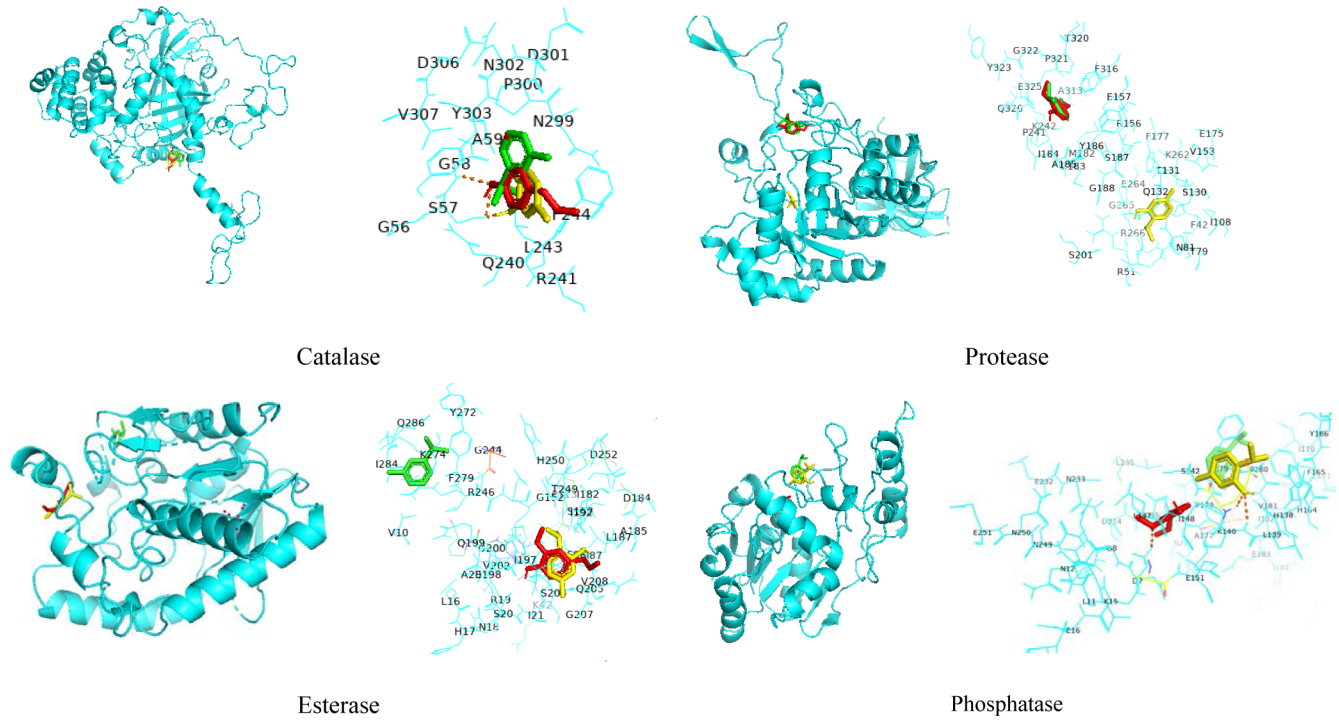

Figure 10. Molecular docking visualization of *Staphylococcus aureus* enzymes binding to key compounds from clove and thyme essential oils. The docking interactions are represented by different colors: eugenol (red), *β*-cymene (green), and thymol (yellow).

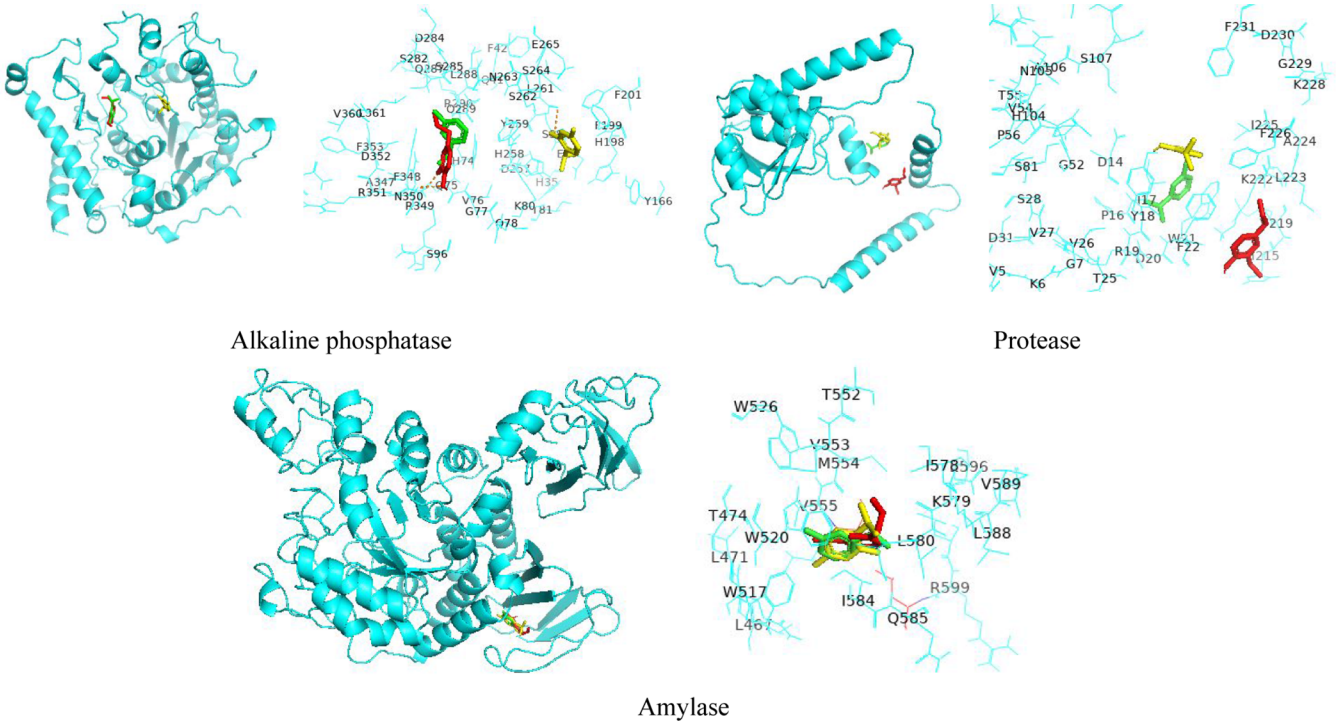

Figure 11. Molecular docking visualization of *Lactiplantibacillus plantarum* enzymes binding to key compounds from clove and thyme essential oils. The docking interactions are represented by different colors: eugenol (red), *β*-cymene (green), and thymol (yellow).

had the highest sensory scores, matching or slightly exceeding the control in overall acceptability (9.6 ± 0.10 vs 9.4 ± 0.10, not significant). Higher concentrations of eucalyptus and myrrh oils showed lower sensory scores than the control, particularly for flavor and mouthfeel, but these differences were not statistically significant ($p > 0.05$).

In another study, El-Kholy and Aamer[48] investigated the sensory quality of ultrafiltrated (UF)-soft cheese fortified with thyme essential oil. The results showed that the addition of TEO significantly enhanced the sensory attributes of the cheese compared to the control sample. The thyme-fortified cheese achieved higher scores in appearance and color (8.60 ± 0.39 vs 8.50 ± 0.41), body and texture (8.55 ± 0.37 vs 8.40 ±

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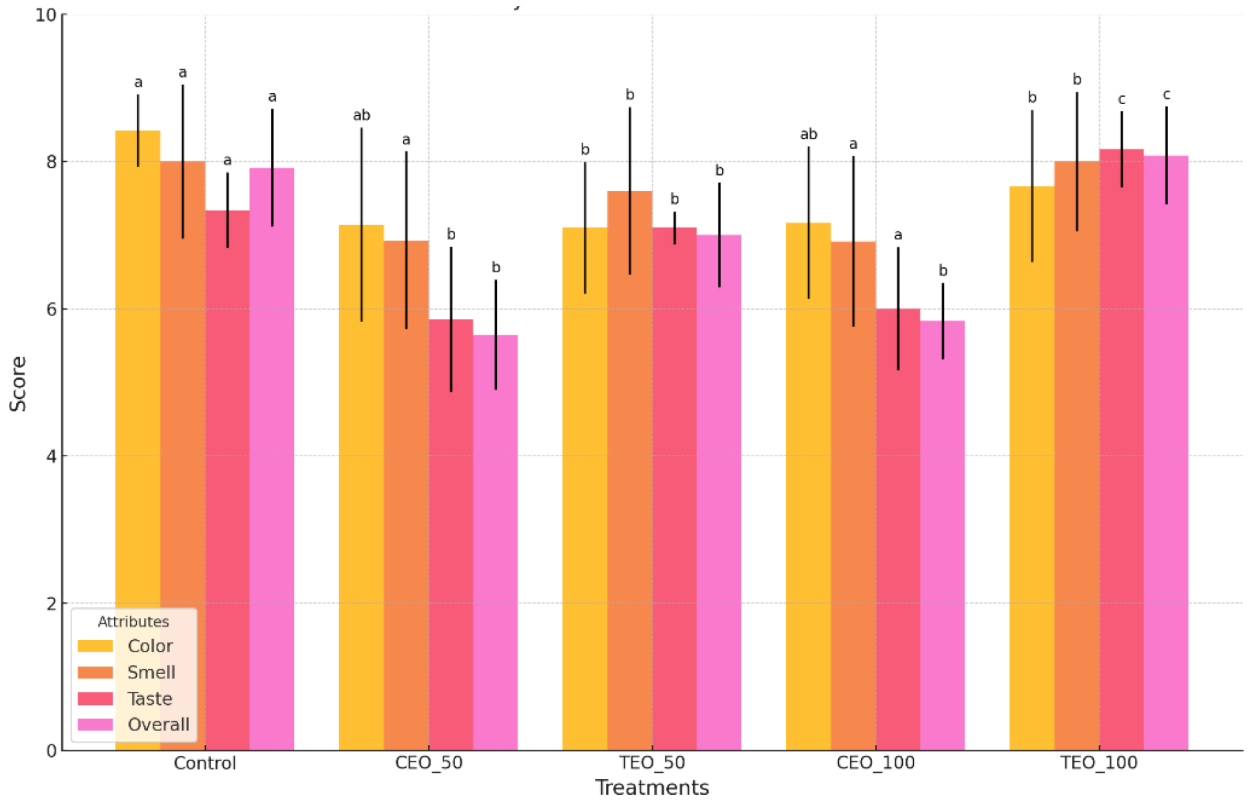

Figure 12. Sensory scores (mean ± SD) of buttermilk samples treated with clove (CEO) and thyme (TEO) essential oils at 50 and 100 μg per 100 mL milk, and the control. Letters indicate significant differences ($p < 0.05$).

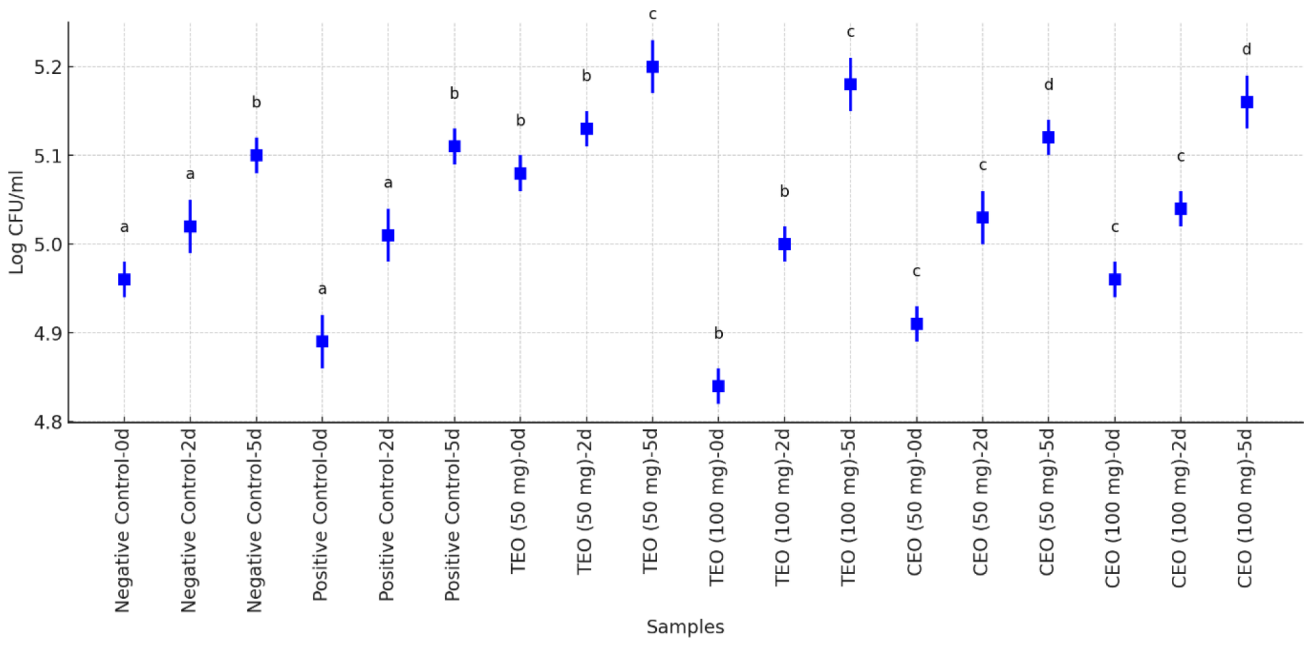

Figure 13. Bacterial growth log CFU/ml over time for different treatments.

0.46), and odor (8.10 ± 0.60 vs 7.50 ± 0.71). The most notable improvement was observed in taste, where the TEO-fortified cheese scored 8.60 ± 0.37 compared to 7.90 ± 0.39 for the control. These enhancements resulted in a higher overall acceptability score for the cheese with TEO (8.50 ± 0.29) compared to the control sample (8.15 ± 0.23).

Antimicrobial Efficacy of Clove and Thyme Essential Oils in Buttermilk. The microbiological quality of buttermilk samples, supplemented with essential oils of clove and thyme, was assessed over a 5-day storage period. LAB, a key beneficial microbial population, were quantified using MRS medium under CO_2-enriched conditions (Figure 13). The analysis focused on evaluating LAB growth in response to EO treatments at concentrations of 50 mg and 100 mg and comparing them with untreated control samples.

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LAB counts across all treatments showed a consistent increase over time, reflecting active microbial proliferation during storage. On Day 0, control samples recorded an average LAB count of 4.96 ± 0.01 log CFU/mL. Samples supplemented with TEO exhibited slightly elevated initial counts of 5.07 ± 0.02 log CFU/mL, while CEO-treated samples displayed averages of 4.94 ± 0.02 log CFU/mL, indicating that the EOs did not inhibit LAB viability at the onset of storage.

By Day 2, LAB counts in all samples increased noticeably. Control samples recorded an average of 5.02 ± 0.01 log CFU/mL, while CEO- and TEO-supplemented samples showed comparable growth, reaching 5.03 ± 0.02 and 5.13 ± 0.01 log CFU/mL, respectively. This trend continued through Day 5, with LAB counts in control samples averaging 5.10 ± 0.01 log CFU/mL. TEO-treated samples reached their highest levels, averaging 5.20 ± 0.02 log CFU/mL, while CEO-treated samples closely followed at 5.16 ± 0.01 log CFU/mL. These results suggest that the presence of EOs supported LAB proliferation throughout the storage period.

Statistical analysis (one-way ANOVA) confirmed no significant differences ($p > 0.05$) in LAB counts between treatments across all time points. This indicates that CEO and TEO supplementation did not adversely affect LAB growth or viability. The data further suggest that the EOs, while potentially influencing early microbial activity, maintained a microbiological quality comparable to untreated control samples over the 5-day period.

In this context, a study explored the antioxidant and antimicrobial properties of thyme and clove essential oils and their application in minced beef.[11] Both EOs exhibited strong antimicrobial activity, inhibiting a range of spoilage and pathogenic bacteria, with clove EO showing higher in vitro antioxidant activity than thyme EO. In minced beef, the EOs effectively inhibited the growth of *Salmonella typhimurium* and *Coliforms*, with clove EO demonstrating superior antimicrobial effects. Additionally, both thyme and clove EOs reduced lipid oxidation in the beef, as indicated by lower thiobarbituric acid values, suggesting their potential as natural preservatives. Importantly, the EOs did not negatively affect the color of the beef, making them suitable for maintaining both the safety and sensory quality of meat products.

In another study, study investigated the use of biodegradable films enriched with clove and thyme essential oils for food packaging applications.[13] The films, made from a poly(lactide)-poly(butylene adipate-co-terephthalate) (PLA−PBAT) blend, were incorporated with essential oils at concentrations of 1, 5, and 10 wt %. The results demonstrated that the films, especially those with clove oil, exhibited significant antimicrobial activity, effectively inhibiting the growth of *Staphylococcus aureus* and *Escherichia coli*, with clove oil films achieving complete bacterial inhibition at 10 wt %. The films also showed strong UV-blocking properties, particularly clove oil composites, which enhanced their potential for preserving food quality and extending shelf life. While the incorporation of essential oils decreased tensile strength, it improved flexibility and hydrophobicity, making the films suitable for active food packaging. This study underscores the potential of natural essential oils in creating environmentally friendly, antimicrobial packaging solutions.

CONCLUSION

This study demonstrates the potential of clove and thyme essential oils as effective natural preservatives, offering antimicrobial, antioxidant, and sensory benefits for buttermilk. The results highlight their multifunctional roles and provide insights into their selective bacterial inhibition mechanisms, which are critical for probiotic-rich food systems.

GC-MS analysis revealed CEO's phenolic-rich profile, dominated by eugenol (73.45%), which underpins its superior antioxidant and broad-spectrum antibacterial efficacy. TEO, characterized by a more diverse composition led by thymol (27.53%) and β-cymene (26.73%), demonstrated moderate antioxidant activity but excelled in enhancing sensory qualities in fortified buttermilk.

CEO exhibited strong antioxidant activity, with EC_{50} values comparable to ascorbic acid and outperforming synthetic antioxidant BHT. Its antibacterial activity was also superior, achieving broader inhibition zones and lower MIC values than TEO, effectively targeting Gram-positive and Gram-negative pathogens. Molecular docking analysis provided mechanistic insights, showing CEO's eugenol and TEO's thymol interact strongly with pathogenic bacterial enzymes, while weaker interactions with beneficial LAB enzymes preserved probiotic viability.

In fortified buttermilk, TEO significantly enhanced aroma, taste, and overall acceptability, particularly at higher concentrations (100 μg/mL), while CEO treatments had a more neutral sensory impact. Both oils supported LAB growth over a 5-day storage period without inhibiting viability, maintaining microbiological quality comparable to the control. Additionally, CEO and TEO extended the microbial shelf life of fortified buttermilk by inhibiting spoilage bacteria while preserving the beneficial microbial population, ensuring product stability throughout the storage period.

LIMITATIONS AND RECOMMENDATIONS FOR FUTURE STUDIES

While the findings are promising, further research can enhance their practical application. Standardizing the composition of CEO and TEO or using well-characterized blends would help ensure consistent efficacy across different batches. Additionally, exploring controlled storage conditions such as temperature and light exposure can provide insights into maintaining the stability and effectiveness of these essential oils during food processing and storage. Pilot-scale studies are recommended to validate the findings in commercial settings, bridging the gap between laboratory experiments and real-world food production. Future research should also focus on synergistic combinations of CEO and TEO with other natural preservatives to boost their stability and performance. Investigating their application in diverse food matrices, including plant-based alternatives, can further broaden their scope. Moreover, encapsulation techniques could safeguard the bioactive compounds during processing, ensuring reliable antimicrobial activity. Finally, evaluating consumer sensory perceptions across various demographics will help refine the use of CEO and TEO in commercial food products, optimizing both functionality and consumer acceptability. These advancements will pave the way for wider adoption of natural essential oils in food preservation.

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Notes

The authors declare no competing financial interest.

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