RESEARCH PAPER

Development of a feed-compatible thymol–carvacrol nanoemulsion with enhanced antibacterial activity against avian pathogenic *Escherichia coli*

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Running title: Thymol–Carvacrol Nanoemulsion Against Poultry Pathogens

Abstract

The emergence of antimicrobial resistance in poultry production has intensified the search for alternative antimicrobial strategies. Phytogenic compounds such as thymol and carvacrol exhibit promising antibacterial properties; however, their practical application in animal production systems is limited by poor aqueous solubility and instability. This study aimed to develop a feed-compatible thymol–carvacrol nanoemulsion and evaluate its physicochemical properties, antibacterial efficacy, antibiofilm activity, cytocompatibility, and storage stability. The nanoemulsion was prepared using high-speed homogenization followed by ultrasonication with Tween 80 as surfactant. Dynamic light scattering analysis showed that the optimized formulation exhibited a mean particle size of 102 ± 3 nm, polydispersity index (PDI) of 0.18 ± 0.01, zeta potential of −28.6 ± 1.7 mV, and encapsulation efficiency of 93.4 ± 1.9%, indicating a stable nanoscale dispersion. Antibacterial activity against avian pathogenic *Escherichia coli* (APEC) and *Salmonella enterica* demonstrated improved efficacy of the nanoemulsion compared with the free compound blend, with MIC values reduced from 125 to 31.25 µg/mL for APEC and from 250 to 62.5 µg/mL for *S. enterica*. The nanoemulsion also showed strong antibiofilm activity, inhibiting biofilm formation by 77 ± 3%, compared with 42 ± 3% for the free compound mixture. Cytocompatibility analysis revealed cell viability above 90% at antimicrobial concentrations near MIC values (≤50 µg/mL), indicating acceptable biocompatibility. Stability studies demonstrated a gradual increase in particle size during 60 days of storage, ranging from 102 to 120 nm at 25°C and 102 to 112 nm at 4°C, with no evidence of phase separation. These findings indicate that thymol–carvacrol nanoemulsion enhances the antimicrobial performance of phytogenic compounds and may serve as a promising feed-compatible antimicrobial delivery system for poultry production.

Keywords: Avian pathogenic *E. coli*, Nanoemulsion, carvacrol, phytogenic feed additive, poultry antimicrobial, thymol

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Introduction

Antimicrobial resistance represents a major challenge for poultry production and animal health worldwide (Morrow 2024). The widespread use of antibiotics in livestock has contributed to the emergence of resistant bacterial strains, including avian pathogenic *Escherichia coli* (APEC), which causes significant economic losses in poultry farming (Hu *et al.* 2022, Rahayu *et al.* 2025).

Phytogenic compounds such as thymol and carvacrol derived from essential oils have been widely studied for their antimicrobial properties and potential application as natural feed additives (Giannenas *et al.* 2012, Gholami-Ahangaran *et al.* 2022). However, their direct use in animal production systems is limited due to poor water solubility, volatility, and rapid degradation under environmental conditions (Suntres *et al.* 2015, Placha *et al.* 2022). In addition, limited studies have evaluated phytogenic nanoemulsions specifically targeting poultry pathogens under storage-relevant conditions.

Nanoemulsion-based delivery systems have recently emerged as a promising strategy to improve the stability, solubility, and bioavailability of hydrophobic bioactive compounds (Chatzidaki and Mitsou 2025, Kumar and Pathak 2025). Nanoemulsions can enhance antimicrobial efficacy by increasing surface area and improving interaction with microbial cell membranes (Shukla *et al.* 2025, Martínez-Guitián *et al.* 2026).

Therefore, the present study aimed to develop a thymol–carvacrol nanoemulsion and evaluate its physicochemical properties, antibacterial activity against poultry-associated pathogens, antibiofilm activity, cytocompatibility, and storage stability as a

potential feed-compatible antimicrobial system. This study is among the first to integrate dual-temperature stability with antimicrobial and antibiofilm evaluation in a poultry-targeted nanoemulsion system.

2. Materials and Methods

2.2 Materials

Thymol and carvacrol were obtained from Sigma-Aldrich (St. Louis, MO, USA). Tween 80 (polysorbate 80) was purchased from Merck (Darmstadt, Germany) and used as a surfactant. All other chemicals were of analytical grade and obtained from standard commercial suppliers. Bacterial strains of avian pathogenic *Escherichia coli* and *Salmonella enterica* were obtained from the microbiology laboratory culture collection of the Department of Biomedical Science, King Faisal University, Saudi Arabia.

2.3 Preparation of nanoemulsion

The nanoemulsion was prepared by dissolving thymol and carvacrol in the oil phase followed by the addition of Tween 80 as surfactant. The mixture was dispersed in distilled water under continuous magnetic stirring using a magnetic stirrer (IKA, Staufen, Germany), followed by pre-homogenization using a high-speed homogenizer (Ultra-Turrax T25, IKA, Staufen, Germany) operated at 10,000 rpm for 5 min. The resulting emulsion was further ultrasonicated for 5 min to obtain a stable nanoemulsion. Figure 1 shows graphical workflow for the preparation of the nanoemulsion.

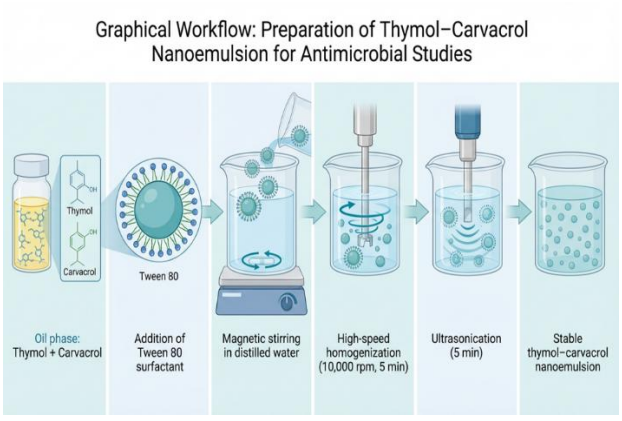

Figure 1. Schematic representation of the preparation workflow of thymol–carvacrol nanoemulsion. The oil phase containing thymol and carvacrol was mixed with Tween 80 surfactant and dispersed in distilled water under magnetic stirring. The mixture was subsequently subjected to high-speed homogenization (10,000 rpm for 5 min) followed by ultrasonication for 5 min to obtain a stable nanoemulsion.

2.4 Characterization of nanoemulsion

Particle size, polydispersity index (PDI), and zeta potential were measured using dynamic light scattering (Zetasizer Nano ZS, Malvern Panalytical, Malvern, UK). Encapsulation efficiency was determined by separating the free compounds using centrifugation (Eppendorf 5804 R, Eppendorf AG, Hamburg, Germany) and calculating the percentage of encapsulated compounds. Table 1 demonstrates formulation composition.

Table 1 Formulation composition

Component	Concentration
Thymol	1%
Carvacrol	1%
Tween 80	2%
Water	balance

2.5 Antibacterial activity

Antibacterial activity was evaluated using agar diffusion and broth microdilution methods to determine inhibition zones, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC).

2.6 Bacterial growth inhibition assay

Bacterial cultures were incubated with different treatments and optical density was measured at 600 nm over 24 h to evaluate growth inhibition kinetics.

2.7 Biofilm inhibition assay

Biofilm formation was assessed using the crystal violet staining method. After incubation with treatments, biofilm biomass was quantified by measuring absorbance at 570 nm.

2.8 Cytocompatibility assay

Cytocompatibility was assessed using an MTT assay in epithelial cells exposed to different concentrations of the nanoemulsion.

2.9 Stability study

Nanoemulsion samples were stored at 4°C and 25°C for over 60 days. Particle size and antimicrobial activity were evaluated periodically.

2.10 Statistical analysis

All experiments were conducted in triplicate. Data were expressed as mean ± standard deviation and analyzed using one-way ANOVA followed by Tukey's post hoc test after confirmation of normal distribution (Shapiro–Wilk test). Statistical significance was considered at $P < 0.05$.

3 Results

3.1 Physicochemical characterization of the nanoemulsion

The thymol–carvacrol nanoemulsion was successfully prepared using high-speed homogenization followed by ultrasonication. Dynamic light scattering analysis showed that the nanoemulsion possessed a small droplet size with narrow distribution, indicating a homogeneous dispersion system. The mean particle size of the drug-loaded nanoemulsion was significantly smaller than that of the blank nanoemulsion (Table 2).

The polydispersity index (PDI) was below 0.3, suggesting a relatively uniform particle size distribution. The zeta potential value indicated good electrostatic stability of the nanoemulsion system, reducing the likelihood of droplet aggregation during storage. Figure 2 demonstrates the comparison of PDI of blank and thymol-carvacrol nanoemulsion.

Encapsulation efficiency analysis demonstrated that a high percentage of thymol and carvacrol was successfully incorporated into the nanoemulsion droplets. The encapsulation efficiency exceeded 90%, indicating effective loading of the active phytogenic compounds.

Overall, these physicochemical characteristics confirm that the developed formulation forms a stable nanoscale delivery system suitable for antimicrobial evaluation.

Table 2. Physicochemical properties of the nanoemulsion

Parameter	Blank Nanoemulsion	Thymol–Carvacrol Nanoemulsion
Particle size (nm)	146 ± 6	102 ± 3
Polydispersity index	0.27 ± 0.02	0.18 ± 0.01
Zeta potential (mV)	−17.9 ± 1.1	−28.6 ± 1.7
Encapsulation efficiency (%)	–	93.4 ± 1.9

Values are expressed as mean ± SD (n = 3). all parameters significantly different (P < 0.01) compared with blank formulation.

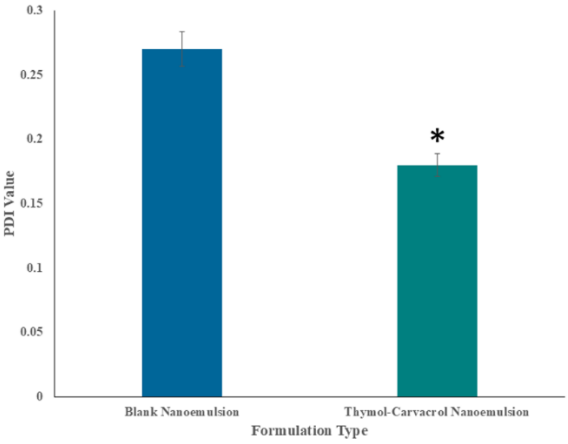

Figure 2. Comparison of the polydispersity index (PDI) of blank nanoemulsion and thymol–carvacrol nanoemulsion formulations. Data are presented as mean ± SD (n = 3). The nanoemulsion containing thymol and carvacrol exhibited a lower PD indicating a more uniform droplet size distribution and improved formulation homogeneity. *(P < 0.01) compared with blank formulation.

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3.2 Antibacterial activity

The antibacterial activity of the thymol–carvacrol nanoemulsion was evaluated against avian pathogenic *Escherichia coli* (APEC) and *Salmonella enterica*. The results demonstrated that the nanoemulsion exhibited stronger antimicrobial effects compared with the free thymol–carvacrol blend. In agar diffusion assays, the nanoemulsion produced larger inhibition zones against both bacterial strains. Similarly, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were lower for the nanoemulsion formulation, indicating improved antibacterial potency. These findings suggest that nanoemulsification enhances the antimicrobial activity of thymol and carvacrol by improving dispersion and facilitating interaction with bacterial cell membranes (Table 3, Figure 3).

Table 3. Antibacterial activity of the formulations

Bacterial strain	Treatment	MIC (µg/mL)	MBC (µg/mL)
E.Coli (APEC)	Free thymol–carvacrol	125	250
E.Coli (APEC)	Nanoemulsion	31.25*	62.5
Salmonella enterica	Free thymol–carvacrol	250	500
Salmonella enterica	Nanoemulsion	62.5*	125

**P < 0.01 compared with Free thymol-carvacrol formulation.*

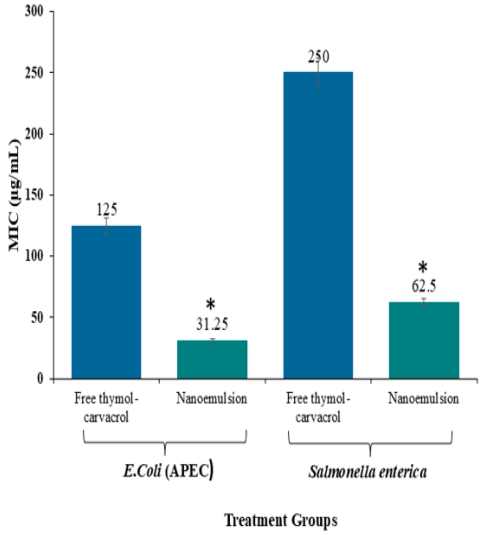

Figure 3. Comparison of minimum inhibitory concentration (MIC) values of free thymol–carvacrol blend and thymol–carvacrol nanoemulsion against avian pathogenic Escherichia coli and Salmonella enterica. Data are expressed as mean ± SD (n = 3).
*P < 0.01 compared with Free thymol-carvacrol formulations.

3.3 Bacterial growth inhibition kinetics

Growth inhibition assays were performed to examine the dynamic antibacterial effects of the nanoemulsion over time (Figure 4). Optical density (OD600) measurements showed that the thymol–carvacrol nanoemulsion reduced bacterial growth by approximately 65–70% at 24 h compared with the untreated control. In addition, the nanoemulsion treatment delayed the onset of the exponential growth phase by approximately 4–6 h, indicating a strong bacteriostatic effect during early incubation. These results demonstrate that nanoemulsification enhances the sustained antibacterial activity of thymol and carvacrol.

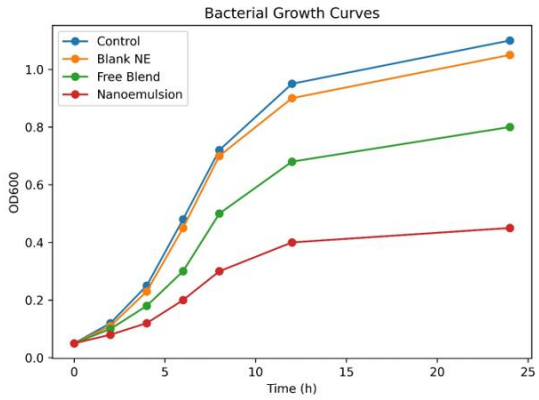

Figure 4. Growth inhibition curves of avian pathogenic *Escherichia coli* over 24 h in the presence of different treatments: control, blank nanoemulsion (Blank NE), free thymol–carvacrol blend (Free Blend), and thymol–carvacrol nanoemulsion (Nanoemulsion). Optical density was measured at 600 nm.

3.4 Biofilm inhibition activity

Biofilm formation plays an important role in bacterial persistence and antimicrobial resistance. The antibiofilm activity of the nanoemulsion was evaluated using crystal violet staining.

The results showed that the thymol–carvacrol nanoemulsion significantly ($P < 0.01$) reduced biofilm biomass compared with both the control and the free compound mixture. The nanoemulsion inhibited biofilm formation by 77 ± 3%, which was approximately 1.8-fold higher than the free compound mixture (42 ± 3%) in APEC cultures, indicating strong antibiofilm potential (Table 4, Figure 5).

The enhanced antibiofilm activity may be attributed to the improved penetration of nanoemulsion droplets into the extracellular polymeric matrix of bacterial biofilms.

Table 4. Biofilm inhibition activity

Treatment	Biofilm inhibition (%)
Control	0
Blank nanoemulsion	6 ± 2
Free thymol–carvacrol	42 ± 3
Nanoemulsion	77 ± 3*

**P < 0.001 vs.control and free blend.*

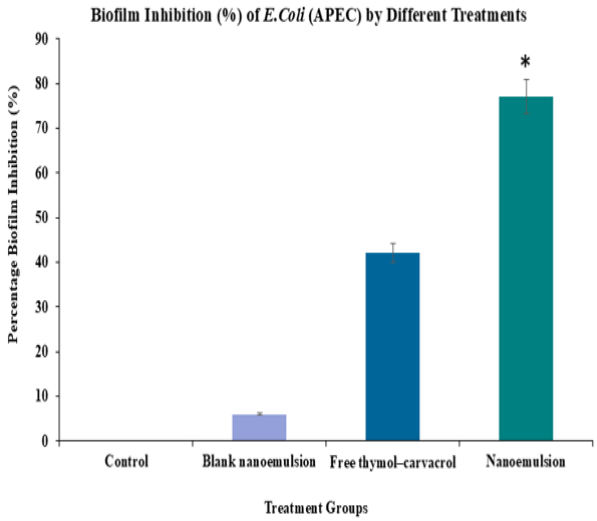

Figure 5. Percentage inhibition of biofilm formation in avian pathogenic *Escherichia coli* after treatment with free thymol–carvacrol blend and thymol–carvacrol nanoemulsion. **P < 0.001 vs.control and free blend.*

3.5 Cytocompatibility evaluation

The cytocompatibility of the nanoemulsion was assessed using an MTT assay in epithelial cells exposed to increasing concentrations of the formulation. The results demonstrated that the nanoemulsion maintained high cell viability at lower concentrations, with more than 90% viability observed at concentrations corresponding to effective antimicrobial level (Table 5, Figure 6). A gradual decrease in cell viability was observed only at higher concentrations. These findings indicate that the nanoemulsion formulation possesses acceptable cytocompatibility within the antimicrobial therapeutic range.

Table 5. Cytocompatibility of nanoemulsion

Concentration (µg/mL)	Cell viability (%)
5	98 ± 2
10	96 ± 2
25	92 ± 3
50	89 ± 2
100	81 ± 4

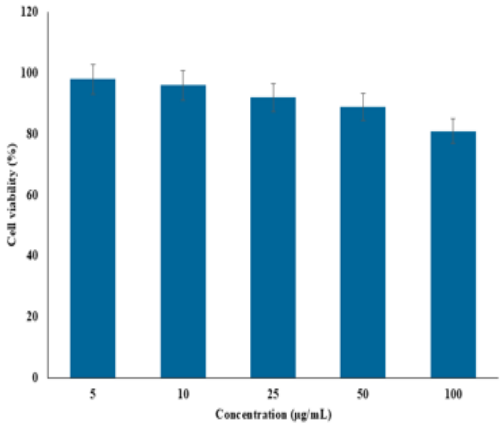

Figure 6. Cell viability of epithelial cells exposed to different concentrations of thymol–carvacrol nanoemulsion as determined by MTT assay.

3.6 Storage stability

The nanoemulsion exhibited temperature-dependent stability. At 25°C, particle size increased from 102 nm to 120 nm over 60 days, representing an approximate 17.6% increase. In contrast, storage at 4°C resulted in a smaller increase from 102 nm to 112 nm (~9.8% increase), indicating improved physical stability under refrigerated conditions. Similarly, the polydispersity index (PDI) increased from 0.18 to 0.25 at 25°C, whereas a lower increase (0.18 to 0.22) was observed at 4°C, reflecting better maintenance of droplet uniformity (Table 6, Figure 7).

Overall, the rate of particle size growth at 25°C was approximately 1.7-fold higher than that observed at 4°C, confirming the strong influence of temperature on nanoemulsion stability.

Table 6. Stability of thymol–carvacrol nanoemulsion at different temperatures

Storage time (days)	Particle size (nm) 25°C	PDI 25°C	Particle size (nm) 4°C	PDI 4°C
0	102	0.18	102	0.18
7	105	0.19	103	0.18
14	108	0.20	105	0.19
21	111	0.21	107	0.20
30	115	0.24	109*	0.21
60	120	0.25	112*	0.22

**P < 0.01 vs. particle size 25°C at 30 and 60 days*

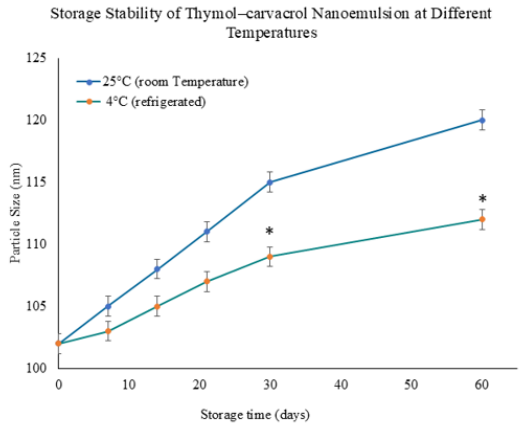

Figure 7. Storage stability of thymol–carvacrol nanoemulsion at different temperatures (4°C and 25°C) over 60 days. Particle size was measured using dynamic light scattering. Data are presented as mean ± SD (n = 3). The nanoemulsion exhibited a gradual increase in particle size during storage, with significantly lower growth observed at 4°C compared with 25°C, indicating improved stability under refrigerated conditions. **P < 0.01 vs. particle size 25°C at 30 and 60 days*

Discussion

The present study demonstrates that a thymol–carvacrol nanoemulsion can be successfully formulated as a stable nanoscale delivery system with enhanced antibacterial activity against poultry-associated pathogens. The formulation exhibited favorable physicochemical characteristics, improved antimicrobial efficacy, strong antibiofilm activity, acceptable cytocompatibility, and temperature-dependent storage stability. These findings collectively support the potential of phytogenic nanoemulsions as alternative antimicrobial strategies in poultry production systems.

The nanoemulsion displayed a mean particle size within the nanoscale range and a relatively low polydispersity index, indicating a uniform droplet distribution and good formulation homogeneity. Nanoemulsions with droplet sizes below 200 nm are known to exhibit improved physical stability and enhanced interaction with microbial membranes due to their large surface area (Barradas and de Holanda e Silva 2021, Garcia *et al.* 2022). In the present study, the small droplet size likely contributed to the improved antibacterial performance observed for the nanoemulsion compared with the free thymol–carvacrol blend. Similar findings have been reported previously which demonstrated that nanoemulsification enhances the antimicrobial activity of essential oils by improving dispersion and facilitating contact with bacterial cells (Jayari *et al.* 2022, Da Silva *et al.* 2023).

The antibacterial assays revealed that the nanoemulsion significantly reduced bacterial growth compared with both untreated controls and free compound treatments. The growth curve analysis showed delayed exponential phase and reduced final biomass in cultures treated with the nanoemulsion, indicating sustained antibacterial activity over time. This observation is consistent with previous reports showing that nanoemulsions can prolong antimicrobial effects through improved solubility and controlled release of active compounds (Ben-Fadhel *et al.* 2024, Mathew *et al.* 2026). Thymol and carvacrol are known to disrupt bacterial membranes by altering lipid bilayer structure, leading to increased permeability and leakage of intracellular contents (Gholami-Ahangaran *et al.* 2022, Wijesundara *et al.* 2022, Latorre *et al.* 2025, Chen *et al.* 2026). The nanoscale delivery system likely enhances these effects by promoting closer interaction between the active compounds and bacterial cell membranes.

In addition to antibacterial activity, the nanoemulsion demonstrated strong antibiofilm effects, reducing biofilm formation significantly compared with the free compound blend. Biofilm-associated bacteria are known to exhibit increased resistance to antimicrobial agents due to the protective extracellular matrix. The enhanced antibiofilm activity observed in this study may be attributed to the ability of nanoemulsion droplets to penetrate biofilm structures and disrupt cell–cell adhesion. Maurya et al. (2021) reported that nanoemulsified essential oils exhibit superior antibiofilm activity compared with their non-encapsulated counterparts, supporting the findings of the present study (Maurya *et al.* 2021). This property is particularly relevant in poultry production systems, where biofilm formation contributes to persistent

contamination in water lines and housing environments.

The cytocompatibility results indicated that the nanoemulsion maintained high cell viability at concentrations effective for antibacterial activity, suggesting an acceptable safety profile. This finding is consistent with previous studies reporting that phytogenic compounds such as thymol and carvacrol are generally recognized as safe and can be used as feed additives (Reis *et al.* 2018, Gholami-Ahangaran *et al.* 2022, Kazempoor *et al.* 2022). The ability to achieve antimicrobial effects without significant cytotoxicity is critical for the development of practical applications in animal production.

A key advancement in the present study is the evaluation of storage stability under different temperature conditions. The nanoemulsion exhibited a gradual increase in particle size over 60 days, indicating some degree of droplet aggregation during storage. However, the extent of this increase was strongly temperature-dependent. At 25°C, particle size increased from 102 nm to 120 nm, whereas storage at 4°C resulted in a smaller increase (102 nm to 112 nm). Similarly, the polydispersity index increased more slowly at 4°C compared with 25°C, indicating better maintenance of droplet uniformity under refrigerated conditions. These findings suggest that lower storage temperatures can significantly improve the physical stability of the nanoemulsion.

The observed temperature-dependent stability is consistent with previous reports indicating that increased temperature can accelerate droplet coalescence and Ostwald ripening in nanoemulsion systems (Kim *et al.* 2016, Trujillo-Cayado *et al.* 2020, Guo *et al.* 2024). Salvia-Trujillo et al. (2015) also reported that essential oil nanoemulsions exhibit improved stability when stored at lower temperatures, supporting the findings of the present study (Salvia-Trujillo *et al.* 2015). The ability to maintain stability under refrigerated conditions is particularly relevant for practical applications, as feed additives or veterinary formulations are often stored under controlled temperature conditions (Subramaniam and Wareing 2016, Akintan *et al.* 2024).

The proposed antibacterial mechanism illustrated in this study provides further insight into the enhanced antimicrobial activity of the nanoemulsion (Figure 8). The interaction of nanoemulsion droplets with the bacterial outer membrane leads to membrane disruption, increased permeability, and leakage of intracellular components such as ions and ATP. In addition, the generation of reactive oxygen species (ROS) contributes to oxidative stress and cellular damage. The nanoemulsion also interferes with bacterial metabolic processes and disrupts biofilm formation, ultimately leading to bacterial cell death. These mechanisms are consistent with the known mode of action of thymol and carvacrol and are further enhanced by nanoemulsion-based delivery systems (Basak and Guha 2018, Kumari *et al.* 2019, Nasra *et al.* 2024).

The novelty of this study lies in the integration of physicochemical characterization, antimicrobial evaluation, antibiofilm activity, cytocompatibility, and dual-temperature stability analysis within a single experimental framework focused on poultry-associated pathogens. While previous studies have investigated essential oil nanoemulsions in food preservation or human health applications (Pathania *et al.* 2018, Aslam *et al.* 2021), relatively limited research has explored their potential use as feed-compatible antimicrobial systems in livestock production. By demonstrating improved antibacterial efficacy and temperature-dependent stability, the present study provides valuable insights into the practical application of phytogenic nanoemulsions in poultry systems.

Nevertheless, some limitations should be acknowledged. The current study was conducted under *in vitro* conditions, and therefore the efficacy of the nanoemulsion in vivo remains to be established. Future studies should evaluate the effects of this formulation in poultry models, including its impact on gut microbiota, pathogen colonization, and animal performance.

Overall, the findings suggest that thymol–carvacrol nanoemulsion represents a promising strategy for enhancing the antimicrobial performance of phytogenic compounds and may contribute to the development of alternative antimicrobial approaches in poultry production systems.

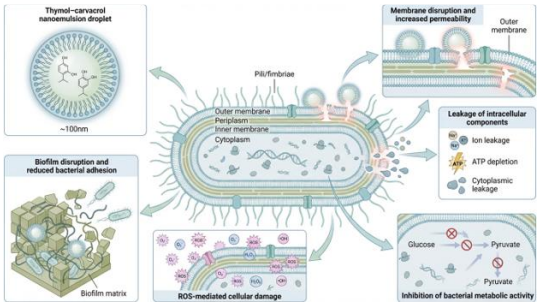

Figure 8. Proposed antibacterial mechanism of thymol–carvacrol nanoemulsion against avian pathogenic *Escherichia coli* (APEC). Nanoemulsion droplets interact with the bacterial outer membrane, leading to membrane disruption and increased permeability. This interaction promotes leakage of intracellular components, including ions and ATP, and induces reactive oxygen species (ROS)–mediated cellular damage. In addition, the nanoemulsion interferes with bacterial metabolic processes and disrupts biofilm formation, ultimately resulting in bacterial cell death.

Conclusion and Practical Implications

The present study demonstrated that a thymol–carvacrol nanoemulsion can be successfully developed as a stable nanoscale delivery system with enhanced antibacterial and antibiofilm activity against poultry-associated bacterial pathogens. The nanoemulsion exhibited favorable physicochemical characteristics, including small droplet size, narrow particle distribution, and high encapsulation efficiency, which contributed to improved dispersion and antimicrobial performance compared with the free phytogenic compounds. In addition, the formulation maintained acceptable cytocompatibility and remained stable during storage, indicating its potential suitability for practical applications.

The enhanced antibacterial activity observed in this study suggests that nanoemulsification may significantly improve the bioavailability and antimicrobial effectiveness of hydrophobic phytogenic compounds such as thymol and carvacrol. Importantly, the strong inhibition of biofilm formation highlights the potential of this nanoemulsion system to reduce persistent bacterial contamination associated with poultry production environments.

From a practical perspective, phytogenic nanoemulsions may represent a promising strategy for developing alternative antimicrobial approaches in livestock production, particularly in the context of increasing restrictions on antibiotic use. Such systems could potentially be incorporated into feed additives, water treatments, or surface sanitation strategies to control pathogenic bacteria in poultry farming systems. Future studies should evaluate the *in vivo* efficacy of this formulation in poultry models to determine its potential effects on pathogen colonization, gut microbiota balance, and overall animal performance.

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Conflict of interest

The Authors declare that there is no conflict of interest.

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