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Research paper

Nanoengineered thyme essential oil as a potent antimicrobial strategy against multidrug-resistant Shiga toxin-producing *Escherichia coli* isolated from raw milk and dairy products

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ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) is a major foodborne pathogen associated with dairy products, while the increasing prevalence of multidrug-resistant (MDR) strains has intensified the need for effective natural antimicrobial alternatives. This study investigated the occurrence, molecular characteristics, antimicrobial resistance, and virulence profiles of STEC isolated from raw milk, yogurt, and Kareish cheese, and evaluated the antibacterial efficacy of a thyme essential oil nanoemulsion. Nineteen *E. coli* isolates were recovered, with isolation rates of 26.66%, 23.33%, and 13.33% from raw milk, yogurt, and Kareish cheese, respectively. Antimicrobial susceptibility testing revealed high resistance to ampicillin (100%), sulfamethoxazole-trimethoprim (94.7%), penicillin (89.5%), and amoxicillin (78.9%), with five isolates exhibiting multidrug-resistant phenotypes. Molecular analysis identified the stx1 gene in 42.1%, 15.78%, and 21.05% of isolates from raw milk, yogurt, and Kareish cheese, respectively, whereas eaeA was detected in only 10.5% of isolates and stx2 was not detected. GC–MS analysis identified thymol (37.5 ± 1.2%) and carvacrol (15.0 ± 0.8%) as the predominant bioactive constituents of thyme essential oil. The nanoemulsion exhibited a mean hydrodynamic diameter of 135.7 ± 23.04 nm, a PDI of 0.251, spherical morphology with a core particle size of 35.45 nm, and significantly enhanced antibacterial activity compared with the free essential oil by reducing the minimum inhibitory concentration from 0.19 ± 0.006 to 0.009 ± 0.0006 mg/mL ($P < 0.001$) and increasing the inhibition zone from 29 ± 1 to 32 ± 1 mm. Scanning electron microscopy confirmed extensive membrane disruption and severe morphological damage in treated bacterial cells. Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Collectively, these findings demonstrate that thyme essential oil nanoemulsion is a promising clean-label antimicrobial strategy for controlling multidrug-resistant STEC and improving the microbiological safety of dairy products.

1. Introduction

Milk and dairy products constitute a vital component of human nutrition due to their high biological value, digestibility, and contribution to essential macro- and micronutrients (Kumar et al., 2026). However, their nutrient-rich composition and high water activity make them particularly susceptible to microbial contamination, posing significant challenges to food safety, quality, and shelf life (El Alami El Hassani et al., 2025). Among dairy products, fermented milk products such as yogurt and fresh soft cheeses, including Kareish cheese, are

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especially vulnerable to contamination by pathogenic and spoilage microorganisms if hygienic practices during production, handling, or storage are compromised (Al-Salman & Haddad, 2026).

Yogurt is a widely consumed fermented dairy product characterized by the presence of beneficial lactic acid bacteria (LAB), which contribute to its nutritional value, sensory attributes, and partial antimicrobial activity (Santos et al., 2025). Despite these advantages, yogurt remains susceptible to contamination by foodborne pathogens, particularly during post-fermentation handling and distribution. The survival of such pathogens in yogurt matrices represents a public health concern, especially in regions where artisanal or small-scale production is prevalent (Wang et al., 2025).

Kareish cheese, a traditional Egyptian fresh soft cheese produced from skimmed milk, is characterized by its high moisture content, low fat levels, minimal salt concentration, and the absence of a ripening stage (Al-Salman & Haddad, 2026). These properties, while nutritionally favorable, create an ideal environment for microbial growth. Numerous studies have reported the frequent isolation of pathogenic bacteria, including antibiotic-resistant strains, from Kareish cheese, highlighting its role as a potential vehicle for foodborne infections. Consequently, improving the microbiological safety of Kareish cheese remains a priority within dairy quality control systems, particularly in developing countries (Izah et al., 2025).

Shiga toxin-producing *Escherichia coli (STEC)*, also known as enterohemorrhagic *E. coli (EHEC),* is a major global public health concern due to its association with a wide spectrum of gastrointestinal illnesses in humans. Clinical manifestations range from mild watery diarrhea to severe bloody diarrhea and may progress to life-threatening complications such as hemolytic uremic syndrome (Egervärn & Flink, 2024). The pathogenicity of *STEC* is primarily attributed to its ability to produce Shiga toxins (*stx1* and *stx2*), also referred to as verotoxins, in addition to other virulence factors such as enterohemolysin *(hly*) and intimin *(eae)*. These virulence determinants play a critical role in the development and severity of *STEC*-associated diseases (Abdulmajeed et al., 2023).

Ruminants, particularly cattle and sheep, serve as the principal reservoirs of *STEC*, harboring the pathogen asymptomatically within their gastrointestinal tracts. These animals can shed *STEC* intermittently and for prolonged periods, with fluctuating bacterial concentrations in their feces (Woube, 2023). Transmission to humans predominantly occurs via the fecal-oral route, either through direct contact with animals or their environments, or through the consumption of contaminated food and water (Zhou & Chen, 2024). Additionally, secondary person-to-person transmission has been reported in domestic and institutional settings (Singha et al., 2023).

The emergence of multidrug-resistant Shiga toxin-producing *Escherichia coli* (STEC) has become a major challenge for dairy safety worldwide (Chowdhury et al., 2025).

Several studies have reported the contamination of raw milk and dairy products with pathogenic *E. coli*, particularly non-*O157:H7 STEC* strains, in both developing and developed countries (Aljasir et al., 2020). Foodborne diseases, therefore, remain a major global public health concern. Conventional food preservation strategies heavily rely on synthetic chemical preservatives, which, despite their effectiveness, are associated with potential adverse health effects, including allergic reactions, carcinogenicity, and mutagenicity (Rathod, Phadke, et al., 2021; Sepahvand et al., 2021; van Breda et al., 2021).

These findings have intensified interest in natural antimicrobial alternatives such as herbs, essential oils (EOs), and plant extracts, which are increasingly investigated for their ability to control food spoilage, inhibit pathogenic microorganisms, and enhance consumer acceptance (Posgay et al., 2022; Sateriale et al., 2022; Yu et al., 2021). Essential oils are aromatic compounds with weak to moderate volatility and are widely considered promising alternatives to synthetic preservatives due to their antimicrobial and antioxidant properties (Busatta et al., 2008; Yu et al., 2021).

However, the application of EOs in food systems—particularly those with high water content and low-fat levels—faces significant challenges owing to their hydrophobic nature, limited solubility, and strong sensory characteristics. At higher concentrations, EOs may impart undesirable flavors and aromas, adversely affecting the nutritional and sensory quality of food products (Giannenas et al., 2020; Jayari et al., 2022). Consequently, although EOs have demonstrated considerable potential, their practical use in food preservation remains limited.

Thyme (*Thymus vulgaris*) extracts, including essential oils obtained from the leaves and flowers, have shown strong antibacterial activity against both Gram-positive and Gram-negative bacteria. Nevertheless, their effects on beneficial microorganisms such as lactic acid bacteria in fermented products must also be considered (Galovičová et al., 2021; Ricardo-Rodrigues et al., 2024). Recently, thyme has gained attention as a valuable source of bioactive compounds with antimicrobial properties (Gedikoglu, 2022). Despite this potential, detailed investigations are still required to evaluate its sensory impact, solubility, hydrophobicity, minimum inhibitory concentrations, interactions with food matrices, and effectiveness against specific target microorganisms (da Silva et al., 2021; Ren et al., 2021; Sepahvand et al., 2021).

GC-MS analysis is employed to identify the active compounds in thyme essential oil, which contribute to its antibacterial, antifungal, antioxidant, and anti-inflammatory properties (Medjadji, Benahmed, & Kadi, 2025).

To overcome these limitations, nanoemulsion-based encapsulation of essential oils has emerged as a promising and rapidly advancing technology. Encapsulation enhances EO stability, improves water solubility, prolongs shelf life, allows controlled release, reduces strong odors, and protects active compounds from oxidation and evaporation while preserving their antimicrobial efficacy (Khairan et al., 2024; Radünz et al., 2020). Moreover, essential oil nanoemulsions have demonstrated stronger antimicrobial activity than free essential oils or coarse emulsions. Although encapsulation is a well-established strategy, the precise mechanisms by which nanoemulsions enhance antimicrobial effectiveness in food systems remain under investigation (Khairan et al., 2024; Radünz et al., 2020).

This study aims to detect and genetically characterize Shiga toxin-producing *Escherichia coli* (*STEC*) isolated from raw milk using PCR and DNA sequencing techniques, and to analyze active components of thyme oil by GC-MS. Furthermore, it seeks to evaluate their antimicrobial sensitivity patterns and assess the inhibitory effects of thyme essential oil (TEO) and thyme nanoemulsion (TNE) against *STEC*.

2. Materials and methods

2.1. Sample collection

The study was conducted from February 2024 to October 2025. A total of 90 random samples of raw milk, yogurt, and Kareish cheese (30 for each) were collected from milk vendors and shops in Assuit Governorate. The samples were transferred to the laboratory under refrigerated conditions (Food Safety Laboratory, Assiut University) and analyzed within a few hours of collection.

2.2. Isolation and identification of E. coli

One milliliter or 1 g of each sample was added to MacConkey enrichment broth (HiMedia, India) and cultured for 24 h at 37°C. Presumptive production samples were streaked over Eosin Methylene Blue (EMB) agar (Oxoid, UK) and incubated for 24 h at 37°C. Presumptive *E. coli* colonies were recognized by their distinctive morphology on EMB agar, appearing as dark, flat colonies with a characteristic green, metallic sheen.

In compliance with the standard protocols outlined in the *Manual of Clinical Microbiology,* confirmation was carried out using traditional biochemical tests, such as catalase. Indole production was detected using Kovacs' reagent (HiMedia, India), methyl red, Voges-Proskauer

(Oxoid, UK), citrate utilization (Oxoid, UK), nitrate reduction (HiMedia, India), urease activity (Oxoid, UK), and carbohydrate fermentation assays (Strockbine et al., 2015).

2.3. Antimicrobial susceptibility testing

The antimicrobial susceptibility testing was performed in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2024) using Mueller–Hinton agar (**Oxoid, UK**). The turbidity of freshly prepared bacterial suspensions was adjusted to approximately 1×10^8 CFU/mL, corresponding to the 0.5 McFarland barium sulfate standard.

The bacterial isolates were tested for susceptibility to eight antimicrobial agents using the disk diffusion method. The antimicrobial disks (Oxoid Limited, Basingstoke, Hampshire, UK) included penicillin (P, 10 IU), streptomycin (S, 10 μg), neomycin (N, 30 μg), tetracycline (TE, 10 μg), ciprofloxacin (CIP, 5 μg), sulphamethoxazole/trimethoprim (SXT, 25 μg), amoxicillin (AML, 30 μg), and ampicillin (AM, 25 μg).

2.4. Detection of Shiga toxin-producing E. coli and intimin using PCR

2.4.1. DNA extraction

Three to four colonies were picked from overnight culture plates and suspended in 100 μL of distilled water in sterile Eppendorf tubes. The suspensions were heated at 95°C for 15 min using a dry block heater, followed by centrifugation at 14,000 rpm for 5 min to pellet cellular debris. The supernatant containing genomic DNA was carefully transferred to properly labeled sterile Eppendorf tubes and stored at −20°C until further use (Ahmed et al., 2009).

Amplification of Shiga toxin (*stx1 and stx2*) and intimin (*eaeA*) genes): Primers were supplied from Metabion (Germany). They have specific sequences and amplify specific products, as illustrated in Table 1.

Uniplex PCR assays were carried out using a thermal cycler (Bio-Rad T100, USA) in a total reaction volume of 20 μL. Each reaction mixture contained 12.3 μL of nuclease-free distilled water, 2 μL of 10× Taq PCR buffer, 1.6 μL of dNTP mixture, 1 μL of forward primer, 1 μL of reverse primer, 2 μL of DNA template, and 0.1 μL of Taq DNA polymerase.

Thermal cycling conditions consisted of an initial denaturation at 96°C for 4 min, followed by 32 cycles of denaturation at 94°C for 20 s, annealing at 50°C for 20 s for *stx1*, and 61°C for 20 s for *eaeA* and *stx2*, with extension at 70°C for 12 s. A final extension step was performed at 72°C for 6 min. The same amplification conditions were applied for *eaeA* and *stx2*, except for the annealing temperature, which was maintained at 61°C.

2.4.2. Analysis of PCR products

The PCR product was separated by gel electrophoresis using 1.5% agarose gel (Applichem, Germany, GmbH) in 1× TBE buffer after staining with ethidium bromide (0.5 μg/mL). A 100-bp DNA ladder (Fermentas, Thermo Scientific, Germany) was used to determine the amplicon sizes. Visualization was performed using ultraviolet illumination (Biometra, Germany), following the method described by Goli et al. (2012). The gels were then photographed using a gel documentation system (Alpha Innotech) and analyzed using the accompanying image analysis software.

2.4.3. Sequencing of Shiga toxin-producing Escherichia coli (STEC)

The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, USA). The BigDye Terminator v3.1 Cycle Sequencing Kit (Perkin-Elmer) was used for sequencing, and products were purified using Centri-Sep spin columns. An Applied Biosystems 3130 Genetic Analyzer (Hitachi, Japan) was used to sequence the DNA. BLAST® analysis (Basic Local Alignment Search Tool) (Altschul et al., 1990) was used to verify sequence identity against GenBank databases.

2.5. Gas chromatography–mass spectrometry (GC-MS) analysis of thyme oil

Thyme essential oil was obtained from the National Research Centre, Cairo, Egypt.

Thyme oil was analyzed using GC-MS (Thermo Scientific TRACE 1300, Waltham, MA, USA) in the Chemistry Laboratory at the Faculty of Science, Assiut University.

Circumstances for GC: Carrier gas: 99.999% pure helium • Constant flow mode flow rate: 0.8 to 1.5 mL/min • Injection volume: 1.0 μL of essential oil diluted in 1:100 n-hexane. The temperature of the injector is 250°C. • A 1:20 split ratio • Oven temperature program: 60°C for 3 min, followed by an increase to 150°C for 2 min at a rate of 4°C per min, then increased to 250°C at a rate of 5 °C/min held for 5 min. MS conditions: Ionization mode: electron impact (EI) at 70 eV. • Mass scan range: 40–550 m/z. • Ion source temperature: 230°C. Interface temperature: 280°C as discussed by El-Kased and El-Kersh (2022).

2.6. Antibacterial effect of thyme essential oil and thyme essential oil nanoemulsion (TNE)

2.6.1. Preparation of thyme nanoemulsion (TNE)

Thyme oil was prepared according to the following method:

At room temperature (25°C), Tween 80 (2% v/v) was dissolved in double-distilled water and stirred using a magnetic stirrer (Daihan, Korea) for 10 min to obtain a homogeneous solution. Thyme essential oil was then slowly added and mixed for 15 min using a direct-drive stirrer. The resulting emulsion was sonicated using an ultrasonic homogenizer (25 kHz, 650 W), according to Moradi and Barati (2019).

Nanoemulsions were prepared at the Nanotechnology Research Unit, Animal Health Research Institute, Assiut, Egypt. Particle size and zeta potential were determined using transmission electron microscopy (TEM; JEOL, USA) and a Zetasizer (3000HS, Malvern Instruments, UK) at a fixed scattering angle of 173°. Measurements were conducted in triplicate at 25°C. Data acquisition and analysis were performed using Zetasizer® software (version 7) (Hassanien et al., 2021).

2.6.2. Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of thyme essential oil and thyme nanoemulsion was determined using a resazurin-based 96-well microdilution assay, according to Elshikh et al. (2016). The MIC was defined as the lowest concentration of the tested antimicrobial agent that completely inhibited visible bacterial growth after 24 h of incubation.

Table 1
Sequences and predicted size of PCR amplification products of the oligonuleotide primers used.

Pathogenic factor	Primer Sequences	Predicted Size (bp)	Reference
Shiga like toxin-1 (stx1)	GAAGAGTCCGTGGGATTACG AGCGATGCAGCTATTAATAA	**130**	Polard et al., 1990
Shiga like toxin-2 (stx2)	ACCGTTTTTCAGATTTT GACACATATACACAGGAGCAGTTTCAGACAGT	**298**	Svenungsson et al., 2000
Intimin (eaeA)	GAAAAACGCTGACCCGCACCTAAAT CACACGAATAAACTGACTAAAAT	**376**	Svenungsson et al., 2000

Bacterial suspensions were prepared following Sanchez et al. (2010) and adjusted to 1×10^8 CFU/mL. A sterile resazurin solution (0.015% w/v) was prepared, vortexed, filter-sterilized through a 0.22 μm membrane filter, and stored at 4°C for up to two weeks.

The assay was performed using a standard two-fold serial dilution technique in sterile 96-well microplates containing Mueller–Hinton broth. The test compounds were serially diluted across the microplate wells, followed by inoculation with the standardized bacterial suspension. Appropriate positive (inoculated broth without treatment) and negative (uninoculated broth) controls were included. Plates were incubated at 37°C for 24 h under aerobic conditions, after which 30 μL/ of resazurin solution was added to each well, and incubation was continued for an additional 2–4 h. Wells that remained blue indicated inhibition of bacterial growth, whereas a color change to pink indicated microbial viability.

MIC endpoints were confirmed by subculturing the contents of wells showing no visible growth onto agar plates to verify the absence of bacterial proliferation. For subsequent analyses, only the lowest effective inhibitory concentration obtained from the MIC assay was selected for the determination of the zone of inhibition.

Thyme oil concentrations tested ranged from 50% to 0.09%, while thyme nanoemulsion concentrations ranged from 5% to 0.009%.

2.6.3. Determination of zone of inhibition (ZOI)

The agar well diffusion method was used to evaluate the antibacterial activity of the tested formulations (Hossain et al., 2022). Briefly, sterile agar plates were inoculated with standardized bacterial suspensions adjusted to 1×10^8 CFU/mL and evenly spread over the surface to ensure uniform growth. Wells of approximately 6–8 mm in diameter were aseptically prepared in the agar medium. The assay was performed using the lowest effective inhibitory concentration previously determined from the resazurin assay, and a fixed volume of thyme essential oil and thyme nanoemulsion, at this concentration, was added to each well.

The plates were incubated under appropriate conditions suitable for bacterial growth, after which the zones of inhibition were measured in millimeters using a calibrated ruler. All experiments were performed in duplicate to ensure the reproducibility of the results.

2.7. Scanning electron microscopy (SEM)

Thyme oil or thyme nanoemulsion was applied to bacterial cultures at selected concentrations, and untreated controls were included. The cells were collected by centrifugation, then fixed for one to 2 h in 2.5% glutaraldehyde in PBS, cleaned, and dehydrated using a graded ethanol series. Samples were sputter-coated with gold, dried in a critical point drier with CO_2, and then subjected to SEM analysis at the proper accelerating voltages and magnifications (Hisada et al., 2023).

2.8. Statistical analysis

Statistical analysis was performed using **Minitab Statistical Software (Version 22.5.1, Minitab LLC, State College, PA, USA).** Data were expressed as mean ± SD of three independent experiments. Differences between treatments were analyzed using one-way ANOVA, followed by Tukey's multiple comparison tests. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Bacterial isolation and antimicrobial susceptibility

Raw milk, yogurt, and Kareish cheese were examined, and *E. coli* strains were isolated at frequencies of 26.66% (8/30), 23.33% (7/30), and 13.33% (4/30), respectively. All isolates were biochemically confirmed, as indicated in Table 2 and Fig. 1. The highest rates of resistance were found for ampicillin (100%), sulphamethoxazole-trimethoprim (94.7%), penicillin (89.5%), and amoxicillin (78.9%). Resistance to neomycin and streptomycin was detected in 31.6% of isolates, while 21.1% were resistant to tetracycline. Only one isolate was resistant to ciprofloxacin. Five isolates were identified as multidrug-resistant (MDR).

Table 2
Antimicrobial resistance profile of *E. coli* isolates (n = 19).

Name of antibiotic	Resistance pattern			
	Sensitive		Resistance	
	No.	%	No.	%
Ciprofloxacin	18	94.7%	1	5.3%
Gentamycin Tetracyline	15	79. %	4	21%
Neomycin	13	68.4%	6	31.6%
Streptomycin	13	68.4%	6	31.6%
Amoxycillin	4	21%	15	78.9%
Penicillin	2	10.5%	17	89.5%
SXT	1	5.3%	18	94.7%
Ampicillin	0	0	19	100%

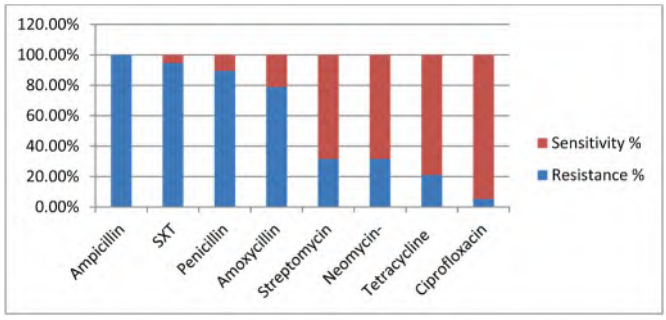


Fig. 1. Antimicrobial resistance profile of *E. coli* isolates (n = 19).

3.2. Molecular characterization of E. coli isolates

Molecular analysis revealed that the nineteen isolated *E. coli* strains from the raw milk, yogurt, and Kareish cheese samples harbored the ***stx1*** gene at frequencies of 42.10**%, 15.78% and 21.05%** respectively (Table 3; Fig. 2). In addition, two isolates one from yogurt and one from Kareish cheese were positive for the ***eaeA*** gene (**10.5%**), as shown in Table 3 and Fig. 3. These isolates exhibited a moderate antimicrobial resistance profile. Notably, none of the examined isolates carried the ***stx2*** gene.

3.3. Sequencing Shiga toxin-producing Escherichia coli (STEC)

Three PCR products obtained from the selected **positive** strains were purified using the QIAquick PCR Purification Kit (Qiagen, France) and sequenced using an ABI 3500 Genetic Analyzer (Applied Biosystems, USA). The obtained sequences were analyzed using the Basic Local

Table 3
Virulence genes of *E. coli* isolates (n = 19).

Source of samples	Number of samples	Number of positive samples	Frequency of virulence gene					
			Stx1		Stx2		eaeA	
			No	%	No	%	No	%
Milk	30	8(26.66% %)	8	42.10	0	0	0	0
Yogurt	30	7(23.33%)	3	15.78	0	0	1	5.26
Kareish cheese	30	4(13.33%)	4	21.05	0	0	1	5.26
Total	90	19(21.2%)	15	78.94	0	0		10.52

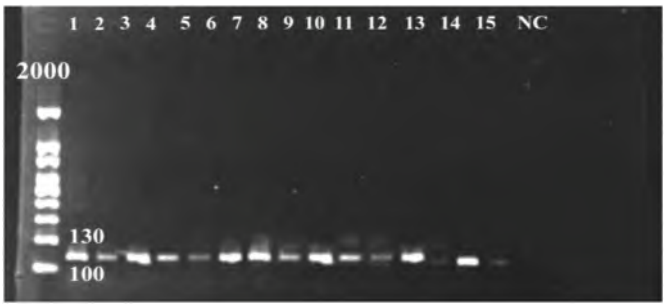


Fig. 2. Agarose gel electrophoresis of amplified *stx-1*,: Marker 100-2000 bp DNA ladder; Lanes1-15 positive amplification of 130 bp of *stx*,lane 16 negative control.

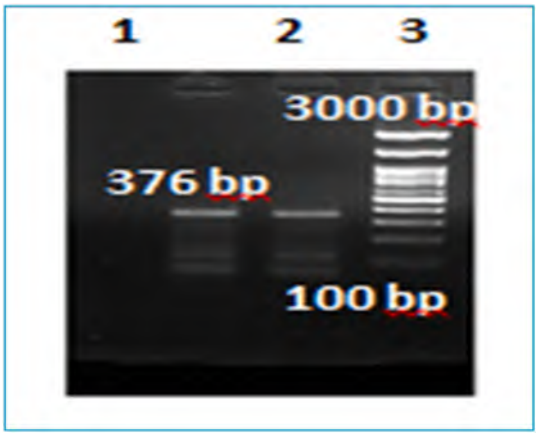


Fig. 3. Agarose gel electrophoresis of amplified *eaeA*: Lane 3: Marker lane 100-3000 bp DNA ladder; Lanes 1&2: positive positive amplification of 376 bp of *eaeA* gene.

Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI).

The sequences were deposited in the GenBank database under accession numbers PP068833 (RM1), PP068834 (RM2), and PP068835 (RM3). Comparative sequence analysis demonstrated high similarity with previously reported pathogenic *E. coli* strains in the GenBank database. Multiple sequence alignments were performed using MUSCLE.

3.4. Gas chromatography–mass spectrometry (GC-MS) analysis of thyme oil

GC-MS analysis was performed in triplicate, and results are presented as relative peak area percentage. The GC-MS analysis of thyme essential oil revealed that thymol is the predominant compound, representing 37.5 ± 1.2%, followed by carvacrol (15.0 ± 0.8%). Other major compounds included p-cymene (11.5 ± 0.5%) and gamma-terpinene (7.5 ± 0.4%). In addition to these major constituents, linalool (5.0 ± 0.3%), β-caryophyllene (3.5 ± 0.2%), α-pinene (2.0 ± 0.1%), and borneol (2.0 ± 0.1%) were also detected at lower concentrations (Table 6).

Table 4
physical analysis of fabricated TNE.

Type of nanoemulsion	Average dynamic size	PDI
TNE	135.7 ± 23.04	0.251

3.5. Physical analysis of fabricated thyme nanoemulsion (TNE)

Table 4 summarizes the average dynamic particle size and polydispersity index (PDI) of the thyme nanoemulsion (TNE). The TNE exhibited a PDI value of **0.251**, indicating a narrow and homogeneous particle-size distribution. Dynamic light scattering analysis revealed an average particle size of **135.7 ± 23.04 nm**, reflecting good stability and consistency of the nanoemulsion.

Transmission electron microscopy (TEM) provided high-resolution visualization of the TNE particles, confirming their nanoscale dimensions and spherical morphology (Fig. 4). The average particle size observed by TEM was **35.45 nm**, indicating a well-defined and uniform nanoemulsion system suitable for antimicrobial applications. The difference between DLS and TEM measurements is attributed to the hydrodynamic diameter measured by DLS, which includes the solvation layer and surface-associated molecules, whereas TEM provides the actual dry-core particle size.

3.6. Determination of minimum inhibitory concentration (MIC) and zone of inhibition (ZOI)

The three virulence gene-positive *E. coli* strains were evaluated for susceptibility to thyme essential oil (TEO) and thyme essential oil nanoemulsion (TNE). As presented in Table 5 and Figs. 5, 6, both TEO and TNE exhibited pronounced antibacterial activity against all tested strains.

Thyme essential oil produced an inhibition zone of **29 ± 1 mm**, with the highest MIC value of 0.19 ± 0.006 mg/mL. In contrast, the thyme nanoemulsion demonstrated superior antibacterial efficacy, recording the largest inhibition zone (**32 ± 1 mm**) with the lowest MIC value (0.009 ± 0.0006 mg/mL) against all three *E. coli* strains (Fig (7)).

Statistical analysis revealed a highly significant difference between thyme essential oil (TEO) and thyme nanoemulsion (TNE) regarding MIC values ($p < 0.001$). The mean MIC of TNE (0.009 ± 0.0006 mg/mL) was significantly lower than that of TEO (0.19 ± 0.006 mg/mL). Regarding the inhibition zone diameter, TNE exhibited a significantly larger inhibition zone (32.0 ± 1.0 mm) than TEO (29.0 ± 1.0 mm)

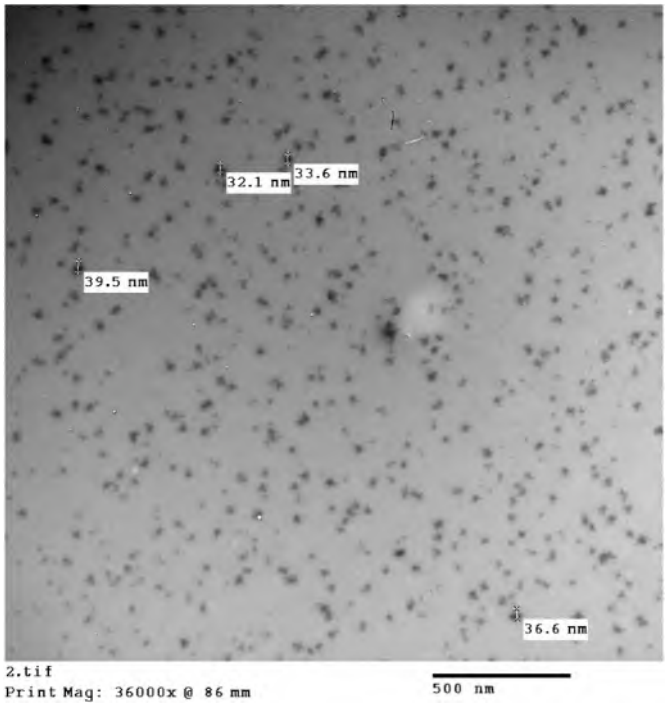


Fig. 4. TEM of TNE with average size 35.45 nm.

Table 5
The MIC and ZOI of thyme oil and thyme oil nanoemulsion against *E. coli* isolates.

Parameter	Thyme oil	TNE	P-value
MIC (mg/mL)	0.19 ± 0.006[a]	0.009 ± 0.0006[b]	<0.001
ZOI (mm)	29.0 ± 1.0[b]	32.0 ± 1.0[a]	0.021

Values are expressed as mean ± SD (n = 3). Different superscript letters indicate significant differences (P < 0.05). Lower MIC values indicate higher antibacterial activity.

Table 6
Major bioactive compounds thyme essential oil identified by GC–MS

Compound	Peak area %	Most effective biological activity
Thymol	37.5 ± 1.2	Strong antimicrobial, Antifungal, Antioxidant
Carvacrol	15.0 ± 0.8	Strong antimicrobial, Antifungal
p-Cymene	11.5 ± 0.5	Antimicrobial (synergistic)
γ-Terpinene	7.5 ± 0.4	Antioxidant
Linalool	5.0 ± 0.3	Antimicrobial, Antifungal
β-Caryophyllene	3.5 ± 0.2	Anti-inflammatory, Antimicrobial
α-Pinene	2.0 ± 0.1	Antimicrobial, Antifungal
Borneol	2.0 ± 0.1	Antioxidant, Antimicrobial

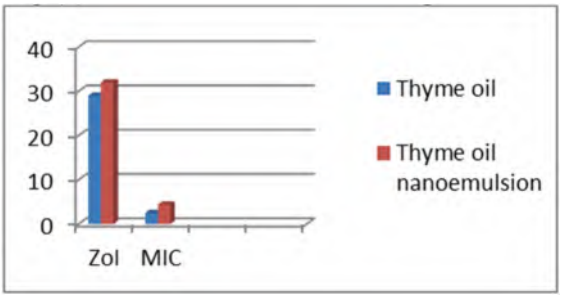


Fig. 5. The MIC and ZOI of thyme oil and thyme oil nanoemulsion against *E. coli* isolates.



Fig. 6. Resazurin-based 96-well plate microdilution assay*1-thyme oil 2-thyme nanoemulsion c-negative control.*

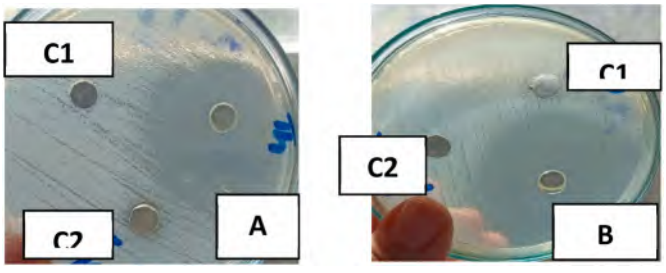


Fig. 7. The zone of inhibition (mm) of thyme oil (A) and thyme nanoemulsion (B) against Shiga toxigenic *Escherichia coli* (STEC).

(p = 0.021).

3.7. Scanning electron microscopy (SEM) imaging

SEM analysis illustrated the antibacterial effects of thyme essential oil and its nanoemulsion on *E. coli* cell morphology (Fig. 8). Untreated *E. coli* cells (Fig. 8A) exhibited intact, smooth, and well-defined cell walls. In contrast, cells treated with thyme essential oil (Fig. 8B) and thyme nanoemulsion (Fig. 8C) showed severe structural damage, including complete cell wall disruption and marked cellular deformation, confirming their potent bactericidal activity.

4. Discussion

Foodborne bacterial infections continue to pose significant global public health challenges, with numerous outbreaks attributed to Shiga toxin-producing *Escherichia coli* (*STEC*) worldwide (Chien et al., 2016; Miszczycha et al., 2013). The major virulence factors of *STEC*, ***stx1*** and ***stx2***, are typically encoded by prophages and can integrate into the *E. coli* genome, enhancing pathogenicity (Martin & Beutin, 2011).

In the present study, the observed microbial findings may reflect regional conditions associated with dairy production in Egypt, including variations in hygienic practices, seasonal fluctuations in raw milk contamination, and potential differences in the prevalence and virulence of local bacterial strains. However, further large-scale epidemiological studies are required to confirm whether these results are representative or influenced by sampling limitations. *E. coli* was isolated from raw milk at a prevalence rate of 26.66%, which closely aligns with reports by Demme and Abegaz (2015) and Farag et al. (2023). Higher isolation rates were documented by Hossain et al. (2017) and Megersa et al. (2019), whereas lower prevalences were reported by Yu et al. (2020) and Elbehiry et al. (2021). The prevalence rate of *E. coli* in yogurt samples **(**23.33%**)** was comparable to findings of Megawer et al. (2020) and Sobeih et al. (2023) but was lower than the rate reported by Aman et al. (2021).

The prevalence rate of *E. coli* isolated from Kareish cheese was 13.33%; this finding was comparable to those reported by Chaleshtori et al. (2017), Hussien et al. (2019), and Alkhafaje et al. (2022), who reported nearly identical values of 11.54%, 16%, and 10%, respectively. Higher prevalence rates of *E. coli were* reported by Ombarak et al. (2016) (74.5%), Sobeih et al. (2023) (30%), and Balabel et al. (2025) (26%).

The widespread use of antimicrobials in livestock production has contributed to the emergence of antimicrobial-resistant *E. coli*, posing serious risks to public health (Burt, 2004; Mohammadi et al., 2013). In the present study, *E. coli* isolates exhibited high resistance to ampicillin, sulphamethoxazole-trimethoprim, penicillin, and amoxicillin, with moderate resistance to neomycin and streptomycin. Five isolates were identified as multidrug-resistant, which is consistent with the findings of earlier studies (Momtaz et al., 2012; Nobili et al., 2016).

The *E. coli* isolates in this investigation showed intermediate resistance to neomycin and streptomycin and high resistance to ampicillin, amoxicillin, penicillin, and sulphamethoxazole-trimethoprim. Consistent with previous studies, five isolates were found to be multidrug-resistant (Momtaz et al., 2012; Nobili et al., 2016).

The detection of the ***stx1*** gene in 42.10**%, 15.78%, and 21.05%** of isolates from raw milk, yogurt, and Kareish cheese, respectively, is consistent with previous studies. In contrast, our findings were higher than those reported by Virpari et al. (2013), Vanitha et al. (2018), Elafify et al., 2020, Farag et al. (2023), and Begum et al. (2026), and lower than those reported by Elbehiry et al. (2021).

The ***eaeA*** gene, which encodes intimin and plays a critical role in intestinal attachment and virulence, was detected in **10.5%** of isolates, one isolate from each yogurt and Kareish cheese.

The obtained results contradict those reported by Chaleshtori et al. (2017) and Mohammed et al. (2021), who found *eae*A-negative *E. coli* strains in Kareish cheese and yogurt. Nevertheless, the *eae*A gene was found more frequently by Latif et al. (2022) and Farag et al. (2023). Vanitha et al. (2018), Hussien et al. (2019), Elafify et al., (2020), and

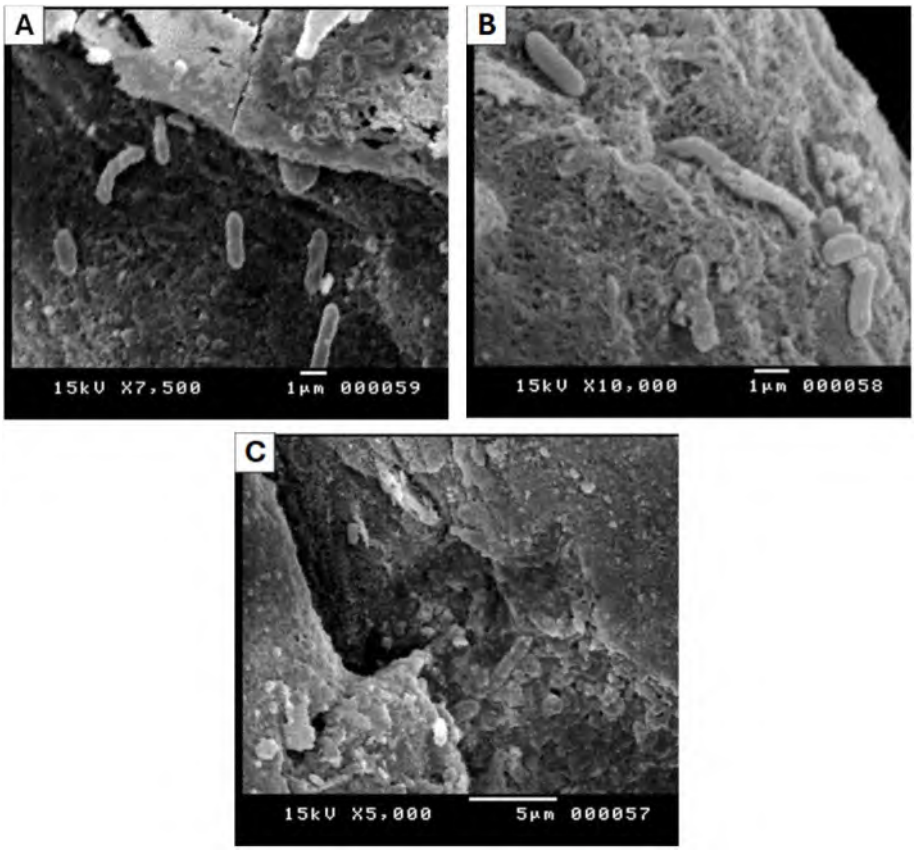


Fig. 8. SEM micrographs of *E. coli* cells: (A) untreated control (×7500, scale bar = 1 µm), (B) thyme oil-treated cells (×10000, scale bar = 1 µm), and (C) thyme nanoemulsion-treated cells (×5000, scale bar = 5 µm). Morphological alterations and cell surface damage were observed in treated cells compared with the untreated control.

Elbehiry et al. (2021) reported different percentages of the *eaeA* gene. These findings highlight the public health risk posed by *non-O157 STEC* strains, which are increasingly associated with severe disease and outbreaks (Jones et al., 2019; Valilis et al., 2018).

None of the 19 isolates in our study harbored the *stx2* gene, which is consistent with the findings of Fetouh et al. (2022), who did not detect the *stx2* gene in the examined strains.

The findings contrast with those reported by Sabry and Laila (2008), who detected the *stx2* gene in 6.1% of raw milk and Kareish cheese, and Virpari et al. (2013), who observed that 22.50% of *E. coli* isolates from milk and dairy products were positive for the *stx2* gene. Similarly, Younis et al. (2021) detected the *stx2* gene in only one isolate (untypeable *E. coli*), whereas Aman et al. (2021) found this gene in three *E. coli* isolates from yogurt. The absence of the *stx2* gene in the current investigation may be attributed to variations in the geographical origin of samples, animal health status, farm hygiene practices, seasonal factors, and the type of dairy products analyzed. The prevalence of *STEC* virulence genes is known to vary significantly among studies and geographical locations.

The GC-MS analysis identified thymol and carvacrol as the primary bioactive constituents of thyme essential oil, fundamentally driving its potent antimicrobial, antifungal, and antioxidant activities. The efficacy of the oil is significantly enhanced by the synergistic interaction between these two phenols, alongside compounds such as p-cymene and gamma-terpinene, which facilitate the neutralization of free radicals and increase microbial membrane permeability. Furthermore, the presence of minor components such as linalool, beta-caryophyllene, and alpha-pinene contributes to the oil's multifaceted biological profile, including its anti-inflammatory effects. The findings are almost identical to those reported by Benameur et al. (2019) and Alkufeidy et al. (2022).

The observed variability in chemical composition reflects the influence of geographical origin and harvest conditions, ultimately highlighting that the oil's therapeutic potential stems from the complex interplay of both major and minor constituents working synergistically.

The antimicrobial efficacy of thyme essential oil and its nanoemulsion observed in this study supports previous findings highlighting their potent antibacterial properties (El-Sayed & El-Sayed, 2020; Rathod, Kulawik, et al., 2021). The superior performance of nanoemulsion may be attributed to enhanced penetration through bacterial membranes and improved physicochemical stability, allowing effective bacterial inhibition at lower concentrations than those required for the free oil (Elsawy et al., 2021; He et al., 2022; Moghimi et al., 2016). In addition, Moghimi et al. (2016) reported that thyme nanoemulsions exhibited 10-fold greater antibacterial activity than the pure essential oil. SEM observations confirmed extensive disruption of the bacterial cell membrane, supporting the proposed bactericidal mechanism of action.

The statistical analysis confirmed that nanoemulsification significantly enhanced the antibacterial activity of thyme essential oil. TNE showed a significantly lower MIC and a significantly larger inhibition zone than TEO. The improved antibacterial activity may be attributed to the reduced droplet size and increased surface area of the nanoemulsion, which enhances the interaction of bioactive compounds with bacterial cells and improves antimicrobial efficacy.

Overall, the results support the application of thyme essential oil nanoemulsions as a promising natural preservative in dairy products, offering enhanced antimicrobial activity and potential sensory advantages over conventional essential oils (Kamal et al., 2024; Yusuf et al., 2025).

5. Conclusion

This study demonstrated the significant occurrence of multidrug-resistant (MDR) Shiga toxin-producing *Escherichia coli* (STEC) harboring stx1 and eaeA virulence genes in raw milk and traditional dairy products, underscoring a critical public health concern. To address this, a stable and homogeneous thyme essential oil nanoemulsion was successfully developed, exhibiting remarkably enhanced antibacterial efficacy against the virulent STEC isolates compared to its free counterpart. Scanning electron microscopy confirmed that this enhanced performance is primarily driven by extensive bacterial membrane disruption and cell wall damage. Collectively, integrating molecular pathogen surveillance with nanotechnology-based delivery offers a promising, sustainable, and clean-label strategy to replace conventional chemical preservatives, thereby improving dairy safety and mitigating the dissemination of antimicrobial-resistant bacteria along the food chain. Future studies should focus on evaluating the efficacy and sensory acceptability of this thyme nanoemulsion within real food matrices during storage, alongside assessing its safety and scalability for industrial food applications.

Ethics approval and consent to participate

No animal studies were conducted under this project; therefore, ethical approval was not required.

Consent for publication

All authors have read and approved the manuscript and agree to its publication.

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CRediT authorship contribution statement

Amany Ibrahim Ali: Methodology, Writing – original draft. **Shaymaa Yusuf:** Data curation, Investigation, Supervision. **Walaa M. Elsherif:** Data curation, Supervision. **Madeha A.H. Darwish:** Investigation, Writing – review & editing. **Enas Abd-Elhafez:** Investigation, Writing – review & editing. **Alshimaa Mohammed Faried Rezk:** Data curation, Methodology, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2026.106761.

Data availability

Data will be made available on request.

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