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Original Research

Thyme Essential Oil: Inhibition of Foodborne Bacteria and Effects on Microbial and Physicochemical Properties of Refrigerated Pork

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Abstract

Background: Overuse of antibiotics and chemical preservatives accelerates the emergence of drug-resistant bacteria and threatens food safety. Thyme essential oil (TEO) is a promising natural alternative; however, the application of TEO in food systems and the antibacterial mechanism underlying its effects require further elucidation. **Objective:** Thus, this study aimed to investigate the antibacterial activity and mode of action of TEO against foodborne bacteria and to evaluate its preservative effects on refrigerated pork. **Methods:** Minimum inhibitory concentrations (MICs) were determined. Membrane integrity was assessed by Propidium iodide (PI) staining under fluorescence microscopy, and DNA binding was analyzed by gel retardation assay. Pork samples were treated with TEO and stored at 4 °C. Microbial growth, bacterial community diversity and structure, pH, drip loss, total volatile basic nitrogen (TVB-N), thiobarbituric acid reactive substances (TBARS), tissue morphology, and sensory quality were monitored during storage. **Results:** TEO exhibited MICs of 0.5–2.5 mg/mL, disrupted bacterial membranes, and showed potential interaction with DNA. TEO also demonstrated clear antioxidant capacity. In refrigerated pork, TEO moderately inhibited microbial growth, enriched bacterial community diversity, influenced microbial structure, maintained lower pH and drip loss, reduced TVB-N and TBARS values, preserved tissue integrity, and improved sensory scores throughout storage. **Conclusions:** TEO exerts antibacterial effects and effectively preserves pork quality by modulating the microbial ecosystem and retarding oxidative spoilage. These findings support its application as a natural and effective food preservative.

Keywords: thyme essential oil; antibacterial activity; spoilage bacteria; preservation

1. Introduction

With the improvement of living standards, there has been an increasing demand for higher meat quality. Fresh pork, a significant source of high-quality animal protein, is prone to microbial contamination and lipid oxidation during storage, leading to spoilage, rancidity, and potential food safety risks. This not only significantly degrades the quality and edible value of the meat but also can cause severe economic losses. Consequently, the preservation of pork and the extension of its shelf life have become key concerns in the meat industry. Currently, preservation technologies for meat products, both domestically and internationally, primarily involve the use of chemical additives, ultraviolet radiation and ultrahigh-pressure treatment [1]. However, the use of chemical preservatives raises safety concerns, radiation technology suffers from low public awareness and ultrahigh-pressure processing can affect the color of pork. Therefore, many researchers are actively seeking advances in new technologies and materials for preserving meat products, such as organic preservatives, which have become promising research interests.

Some plant essential oils are widely recognized as safe substances within certain dosage ranges and have attracted significant attention for their potent antibacterial activity and beneficial antioxidant effects [2]. These oils can be extracted from various parts of plants, such as flowers, leaves, roots, bark, fruits, and seeds [3]. Most plant essential oils have a strong aroma and appear as oily liquids at room temperature. They consist of complex mixtures of terpenes, aromatic compounds, aliphatic compounds, nitrogen- and sulfur-containing compounds, and other substances [4,5,6]. The antibacterial effect of these oils is related to their components, contents, functional groups and synergistic effects between different components, which collectively contribute to their broad-spectrum antibacterial activity [7]. A large number of studies have shown that plant essential oils exhibit excellent antibacterial effects against both Gram-positive bacteria (such as *Staphylococcus aureus* and *Bacillus subtilis*) and Gram-negative bacteria (including *Escherichia coli*, *Salmonella*, and *Helicobacter pylori*) [8,9]. Furthermore, these essential oils also possess strong antifungal activity.

Thymus mongolicus Ronniger is a perennial herbaceous plant of the *Lamiaceae* family. It was recognized as a food spice by the International Organization for Standardization in 1970 and has gained recognition worldwide. Thyme essential oil (TEO), obtained through steam distillation of stems and leaves, has antibacterial and antiseptic activities as well as antioxidant and antitumor properties [10,11]. In addition, it possesses various pharmacological

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properties, including anti-inflammatory effects and the ability to alleviate oxidative stress in tissues and organs [4,12]. Thyme holds significant value due to its edibility, medicinal attributes, and chemical applications. As a culinary enhancer, it also can be used in multiple dishes including meat sauce, sausages, processed meats, and pickles. Furthermore, it is used for flavoring, as a nectar source, for ornamental purposes, and in environmental protection. Therefore, thyme is a promising aromatic plant with potential applications in the food industry, particularly as a natural food preservative. Boskovic et al. [13] suggested that TEO and oregano essential oil have strong antibacterial effects on foodborne microorganisms, including *Salmonella enteritidis*, *Staphylococcus aureus*, *Escherichia coli* and *Bacillus cereus*. However, there is currently a lack of research focused on the antibacterial mechanism of thyme essential oil against foodborne bacteria and its preservative efficacy during refrigerated pork storage. Therefore, the purpose of this study was to investigate the antibacterial activity and action of TEO against common foodborne pathogens, evaluate the effect of TEO on the microbial quality and physical and chemical properties of chilled pork.

2. Materials and Methods

2.1 Thymus Essential Oils, Bacterial Strains, and Chemical Reagents

Thymus essential oil (thymol >40%) was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (catalog number: S26577, Shanghai, China). The essential oil stock solution was prepared by dispersed in 0.4% Tween-80 solution. The bacterial strains used in this study were stored in the laboratory. All the chemical reagents used in this study were obtained from Solarbio (Beijing, China) unless otherwise stated. There were no animal subjects in this study, and the pork used in current study were purchased from the supermarket of Binzhou city (Shandong province, China).

2.2 Antimicrobial Activity and Action of TEO

2.2.1 MIC and MBC

The antimicrobial spectrum of TEO was determined using the microbroth dilution method as previous study [14] with appropriate modifications. TEO stock solutions were prepared at three different concentrations (8, 10, and 12 mg/mL) to ensure that the subsequent serial dilutions could adequately cover the expected minimum inhibitory concentrations (MIC) ranges for all tested strains. Specifically, two-fold serial dilutions of the samples were prepared in 96-well plates containing nutrient broth to obtain a final concentration range of 0.25–12 mg/mL. Then 100 µL bacterial suspension was inoculated into each well, adjusting the final bacterial density to approximately 5×10^5 CFU/mL. A positive control (Kanamycin) and a negative control (sterile medium) were included. Following incubation at 37 °C for 18 h, the MIC was defined as the lowest concentration at which the OD_{600} nm approached zero, with the values corrected by subtracting the absorbance of the corresponding emulsion-containing blank wells to eliminate background turbidity. To determine the minimum bactericidal concentrations (MBC), 100 µL aliquots from the wells of the 96-well plate that exhibited no visible bacterial growth, were spread onto agar plates, and incubated at 37 °C for 18 h, after which the colonies were enumerated. MBC values were the lowest concentrations that could kill 99.9% of the treated bacteria detected by plate counting methods.

2.2.2 Propidium Iodide (PI) Staining

The permeability of the bacterial cell membranes was investigated using fluorescence microscopy in this study. Some modifications were made to the research methods previously used [15]. Bacterial cells in the exponential phase were incubated with thyme essential oil at 37 °C for 1 hour. Subsequently, the cells were treated with a solution of PI at a concentration of 1 mg/mL for another hour at the same temperature. After being washed three times with phosphate-buffered saline (PBS) buffer, the cells stained with PI were immobilized on a glass slide and observed by utilizing a fluorescence microscope equipment (Eclipse 80i; Nikon, Tokyo, Japan).

2.2.3 DNA Binding Analysis

The potential binding of the TEO to the bacterial genomic DNA were investigated by using Gel retardation test assays as described by previous research [15]. In this study, approximately 1.0 µg of bacterial genomic DNA was combined with TEO at different mass ratios. Following this, the combinations were placed in an incubator at 37 °C for a duration of 60 minutes. The movement of the genetic material was then analyzed using electrophoresis on a 1% agarose gel.

2.2.4 Antioxidant Activity Assay

The antioxidant properties of the TEO were evaluated by examining their ability to scavenge various types of free radicals, including 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), which were used by previous study [16].

2.2.5 Measurement of Nucleic Acid Release

The release of nucleic acid from *S. typhimurium* ATCC 14028, *E. coli* ATCC 25922, *L. monocytogenes* ATCC 19115, and *P. aeruginosa* ATCC 9027, was measured using the method reported by previous research [17]. Briefly, bacterial cells in the exponential growth phase were treated with the test samples at 37 °C for 5 h, followed by centrifugation at 10,000 ×g for 5 min to remove cell debris. The supernatant was collected, and its absorbance at 260 nm was measured using Thermo Scientific™ BioMate™ 160 UV-Visible Spectrophotometer.

2.3 Application of TEO to Refrigerated Pork Storage

2.3.1 Microbial Growth Analysis

The muscles of pig hindlimbs utilized in this investigation were acquired from markets in Binzhou City. Before the application of TEO, the pork samples were sliced into consistent measurements of 4 cm × 4 cm × 2 cm (15 grams each). Samples were then soaked in TEO of varying concentrations for 5 minutes. For storage, the pork samples were wrapped with polyethylene (PE) plastic film and kept at 4 °C. Regular collection of samples was conducted for subsequent analysis. To investigate the proliferation of microorganisms in pork, samples were immersed in 30 mL of sterile 0.9% saline solution. A 10-fold reduction in concentration was then carried out, followed by the plating of 200 µL of the diluted sample onto counting agar. The plates were incubated at 37 °C for 24 hours. The quantification of microbial colonies on the agar plates allowed for the determination of microbial growth in the pork samples [18]. All of these experimental procedures were executed three times (Biological replicates) to ensure the reliability of the results.

2.3.2 Microbial Community Analysis

For each experimental group, three independent biological replicates were prepared. Approximately 15 g of pork was mixed with 50 mL of phosphate-buffered saline (PBS) solution, and after immersion for 5 minutes, the bacterial suspension was filtered through three layers of sterile gauze to remove tissue debris. The filtrate was then centrifuged at 12,000 rpm for 10 minutes at 4 °C to collect bacterial pellets, which were subsequently stored at –80 °C until further processing. Total genomic DNA was extracted from the collected bacterial pellets using a commercial DNA extraction kit according to the manufacturer's instructions, and the hypervariable region V3–V4 of the bacterial 16S rRNA gene was amplified using the specific primer pair 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The purified amplicon library was subjected to paired-end sequencing on the Illumina NovaSeq 6000 platform at Biomarker Technologies Co., Ltd. (Beijing, China). Raw sequencing data were processed using the BMK Cloud platform following the standard microbial diversity analysis pipeline, which involved removing low-quality reads (average Phred score < Q20), reads containing ambiguous bases, and reads with adapter contamination, followed by assembly and further filtering to obtain clean tags. The high-quality sequences were clustered into operational taxonomic units (OTUs) at a 97% similarity threshold using UPARSE, and taxonomic classification of OTU representative sequences was performed against the SILVA database (version 138.1, Leibniz Institute DSMZ, Bremen, Germany) using the Ribosomal Database Project (RDP) Classifier with a confidence threshold of 0.7. Alpha-diversity indices, including Chao1, observed OTUs, Shannon, and Simpson, were calculated to estimate community richness and diversity, while Beta-diversity was evaluated based on Bray–Curtis dissimilarity and UniFrac distances and visualized via Principal Component Analysis (PCA). To determine whether the observed community structural differences were statistically significant, PERMANOVA (permutational multivariate analysis of variance) with 999 permutations was performed, with pairwise PERMANOVA and False Discovery Rate (FDR) correction applied when significant overall effects were detected. For statistical evaluation of microbial community differences, one-way Analysis of Variance (ANOVA) followed by Duncan's multiple range test was performed at each individual time point to compare treatment groups, as samples from different storage times were independently collected from separate batches rather than repeatedly measured from the same biological unit, and significance was set at $p < 0.05$. All results are presented as mean ± standard deviation (SD) from three independent biological replicates, and all analytical procedures described above are consistent with previously published studies [15].

2.3.3 pH and Drip Loss

Each pork sample (10 g) was blended with 100 mL of sterile distilled water (1:10, w/v). Use a pH meter to assess the pH level of the mixture obtained. To calculate the drip loss coefficient (%), use the formula: (initial mass – final mass) / initial mass × 100%. Each experiment was conducted in triplicate (Biological replicates) to ensure accuracy.

2.3.4 Determination of TVB-N Content and TBARS Values

The TVB-N content was determined using the Kjeldahl nitrogen determination method with reference to the national standard of the People's Republic of China (GB 5009.237-2016). Pork samples were analyzed for TBARS values by means of a UV-visible spectrophotometer (Nanjing Detie Instrument Co., Ltd., Nanjing, China), with the optical density being recorded at 532 nm. The concentration of malondialdehyde (MDA) in the pork was calculated using a standard curve of 1,1,3,3-tetramethoxypropane. The results were expressed as TBARS values (mg MDA equivalent/100 g pork). All experiments were performed in triplicate to verify the accuracy of the findings.

2.3.5 Pork Tissue Morphology Analysis

Pork tissues were fixed in 4% paraformaldehyde and subsequently embedded in paraffin. The sections, measuring 2 µm in thickness, were subjected to staining with hematoxylin and eosin (H&E). Microscopic images were captured using a microscope (Olympus BX51, Japan).

2.3.6 Sensory Quality Evaluation of the Pork During Cold Storage

The sensory attributes of pork samples, such as color, smell, texture, and overall appearance, were evaluated using established quality parameters as shown in previous study [16]. 20 trained panelists participated in the sensory evaluation. All assessments were performed blind, with each sample coded with a random three-digit number to prevent bias. The presentation order was randomized across panelists to eliminate order effects. Each sample was evaluated independently three times, and the mean scores were used for statistical analysis. All panelists provided written informed consent. Since this study involved only sensory testing of edible meat products without any health-related intervention, formal ethical approval was not required. Subsequently, the samples were graded on a scale ranging from 1 to 10. Excellent and very acceptable scores are between 8 and 10, and good and acceptable scores are between 6 and 8. The poor and unacceptable grades range from 4 to 6; the very poor and very unacceptable grades range from 0 to 4.

Table 1. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of thyme essential oil against different Gram-positive and Gram-negative bacteria.

Bacterial strain	MIC (mg/mL)	MBC (mg/mL)
Gram-positive		
Listeria monocytogenes ATCC 19115	0.5	1.0
Bacillus subtilis ATCC 6633	1.0	2.0
Bacillus subtilis AHU 1035	0.5	1.5
Staphylococcus aureus ATCC 25923	1.0	2.0
Gram-negative		
Cronobacter sakazakii ATCC 29544	2.0	5.0
Escherichia coli ATCC 25922	2.5	6.0
Salmonella typhimurium ATCC 14028	1.5	4.0
Pseudomonas aeruginosa ATCC 9027	1.0	6.0

MIC: Minimum inhibitory concentrations.

MBC: Minimum bactericidal concentrations.

2.4 Statistical Analysis

Statistical analysis of the collected data was carried out by using GraphPad Prism 7.0 software (GraphPad Software, La Jolla, CA, USA). The acquired results were subjected to statistical examination using one-way ANOVA and Duncan's multiple range test. Additionally, the mean ± standard deviation was used to display all the data in the manuscript. To establish statistical significance, a p value less than 0.05 was used. Three independent biological replicates were performed.

3. Results

3.1 Antimicrobial Activity and Mechanism of TEO

3.1.1 MIC and MBC of TEO

The MIC and MBC of TEO against 8 types of foodborne pathogenic bacteria, including both Gram-positive and Gram-negative strains, was determined. Table 1 lists the antimicrobial activities of the TEO against the two bacterial species. The minimum concentration needed to inhibit the growth of different strains varies, ranging from 0.5 to 2.5 mg/mL. The minimum bactericidal concentration was found to be 1.0–6.0 mg/mL. Essential oils are well-recognized for their broad-spectrum antimicrobial activity against various foodborne pathogens, including both Gram-positive and Gram-negative bacteria and demonstrating their broad-spectrum antimicrobial properties [19]. Numerous previous studies have reported the MIC and MBC values of TEO against various foodborne pathogens. Collectively, the MIC ranges reported for Gram-positive bacteria fall between 0.156 µg/mL and 0.5 mg/mL, while those for Gram-negative bacteria range from 0.078 µg/mL to 0.5 mg/mL [14,20]. Compared with these earlier reports, the MIC values observed in our study are relatively higher, which is not unexpected. TEO is a complex mixture of multiple bioactive constituents and variations in the relative abundance of these components can significantly influence its antimicrobial potency. The antibacterial impact of essential oil components relies primarily on the lipophilicity of the hydrocarbon structure and the hydrophilicity of the functional groups. The general recognized order of action of the essential oil components was as follows: phenols > aldehydes > ketones > alcohols > ethers > hydrocarbons [21]. Thyme essential oil used in this study contains thymol, a monoterpene with a phenolic ring as illustrated in the manufacturer. Additionally, differences in the viability and intrinsic tolerance of various bacterial strains to essential oils may also contribute to the observed discrepancies. Nevertheless, the fundamental conclusion and overall trend of our findings remain consistent with those of previous investigations, namely that TEO exhibits superior inhibitory activity against Gram-positive bacteria compared to Gram-negative counterparts [14,19,21]. This differential susceptibility is primarily attributable to the permeability barrier conferred by the outer lipopolysaccharide (LPS) layer in Gram-negative bacteria. Specifically, the LPS layer forms a hydrophilic outer surface that acts as an effective permeability barrier, preventing large molecules and hydrophobic compounds, such as many essential oil components, from penetrating the inner cytoplasmic membrane. As a result, Gram-negative bacteria generally exhibit greater resistance

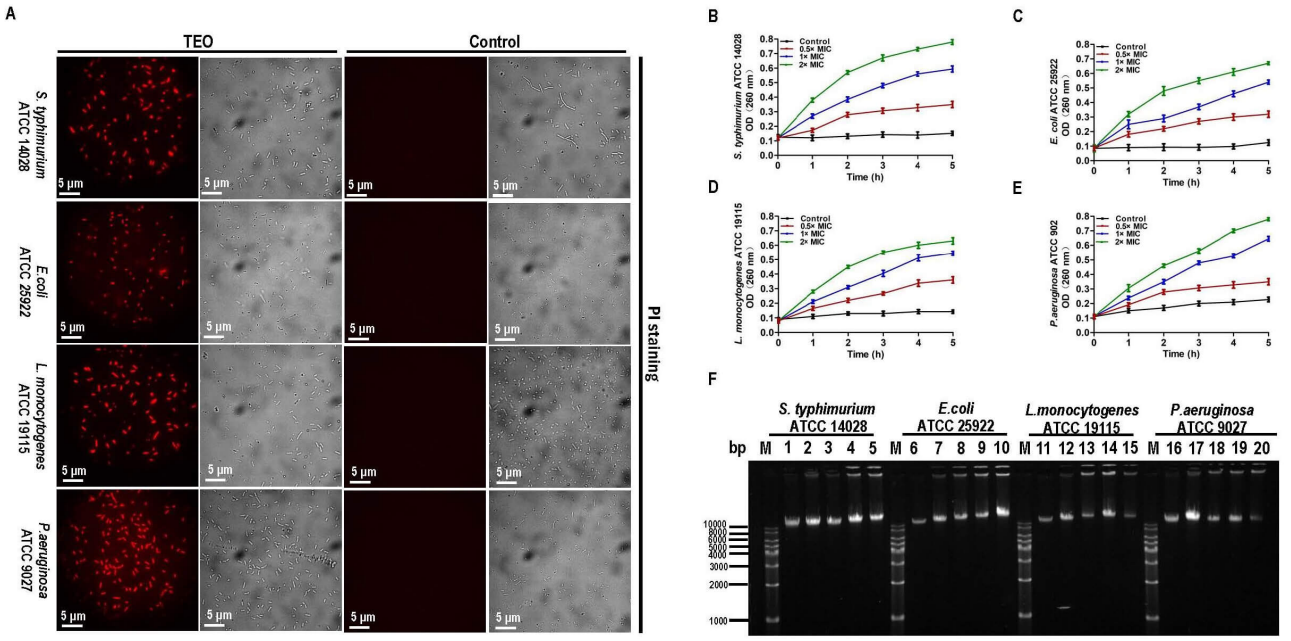


Fig. 1. PI staining and cell permeabilization effects of bacteria treated with thyme essential oil. (A) PI staining of bacterial cells, including *S. typhimurium* ATCC 14028, *E. coli* ATCC 25922, *L. monocytogenes* ATCC 19115, and *P. aeruginosa* ATCC 9027, treated with thyme essential oil. Scale bars: 5 μm. (B–E) OD_{260} values of cell suspensions, including *S. typhimurium* ATCC 14028, *E. coli* ATCC 25922, *L. monocytogenes* ATCC 19115, and *P. aeruginosa* ATCC 9027, treated with thyme essential oil. (F) A gel retardation assay was performed on bacterial genomes treated with different concentrations of thyme essential oil. Lane M displays the DNA marker. Lanes 1–5 show the genome of *S. typhimurium* ATCC 14028 treated with 0, 1.0, 2.0, 4.0, or 8.0 mg/mL thyme essential oil. Lanes 6–10 show the genome of *E. coli* ATCC 25922 treated with the same concentrations of thyme essential oil. Lanes 11–15 display the genome of *L. monocytogenes* ATCC 19115 treated with 0, 1, 2, 4, or 8 mg/mL thyme essential oil. Finally, lanes 16–20 show the genome of *P. aeruginosa* ATCC 9027 treated with 0, 2, 4, 8, or 10 mg/mL thyme essential oil. PI, Propidium iodide.

to hydrophobic antimicrobial agents compared to Gram-positive bacteria, which lack this outer membrane structure. The results of this study provide compelling evidence of the potent antibacterial properties of TEO.

3.1.2 Disruption of Bacterial Cell Membrane by TEO

In order to study the bactericidal mechanism of thyme essential oil, PI staining was used to observe bacterial invasion, and bacterial invasion was observed under a fluorescence microscope. After TEO treatment, a noticeable red fluorescence was observed in the bacterial cells under fluorescence microscopy, while no fluorescence signal was detected in the control group (Fig. 1A). These results indicate that TEO treatment compromised the integrity of the bacterial cell membrane, allowing PI to penetrate and bind to nucleic acids. UV spectroscopy is a commonly used method for determining the amount of protein and nucleic acid. This method involves analyzing the absorption peaks at wavelengths of 260 nm by Thermo Scientific™ BioMate™ 160 UV-Visible Spectrophotometer, which are considered important indicators. The results confirmed that the absorbance at 260 nm increased over time in the bacterial culture treated with different concentrations of TEO, with a higher concentration leading to a faster increase in the absorbance (Fig. 1B–E). To further evaluate the interaction between bacterial DNA and TEO, we conducted DNA gel retardation experiments to examine the binding ability of TEO to bacterial genomes. TEO treatment resulted in reduced DNA mobility in gel electrophoresis, suggesting a possible interaction with genomic DNA (Fig. 1F). PI staining results suggested that TEO treatment disrupted the integrity of the bacterial cell membrane, leading to the leakage of intracellular contents. These results suggest that TEO exerts its antibacterial activity by disrupting the integrity of the bacterial cell membrane. In addition, the DNA gel retardation assay showed that TEO components can interact with purified bacterial DNA *in vitro*, implying a possible additional action that may involve interference with DNA function. Collectively, these findings suggest that the bactericidal activity of TEO is primarily attributable to membrane disruption, with a possible additional contribution through interaction with intracellular DNA.

3.2 Application of TEO in Pork Cold Refrigeration

3.2.1 Microbial Growth Determination

According to Fig. 2A, the total viable cell count (TVC) of the control pork samples significantly increased from day 0 to day 3. In contrast, the TVC of pork samples

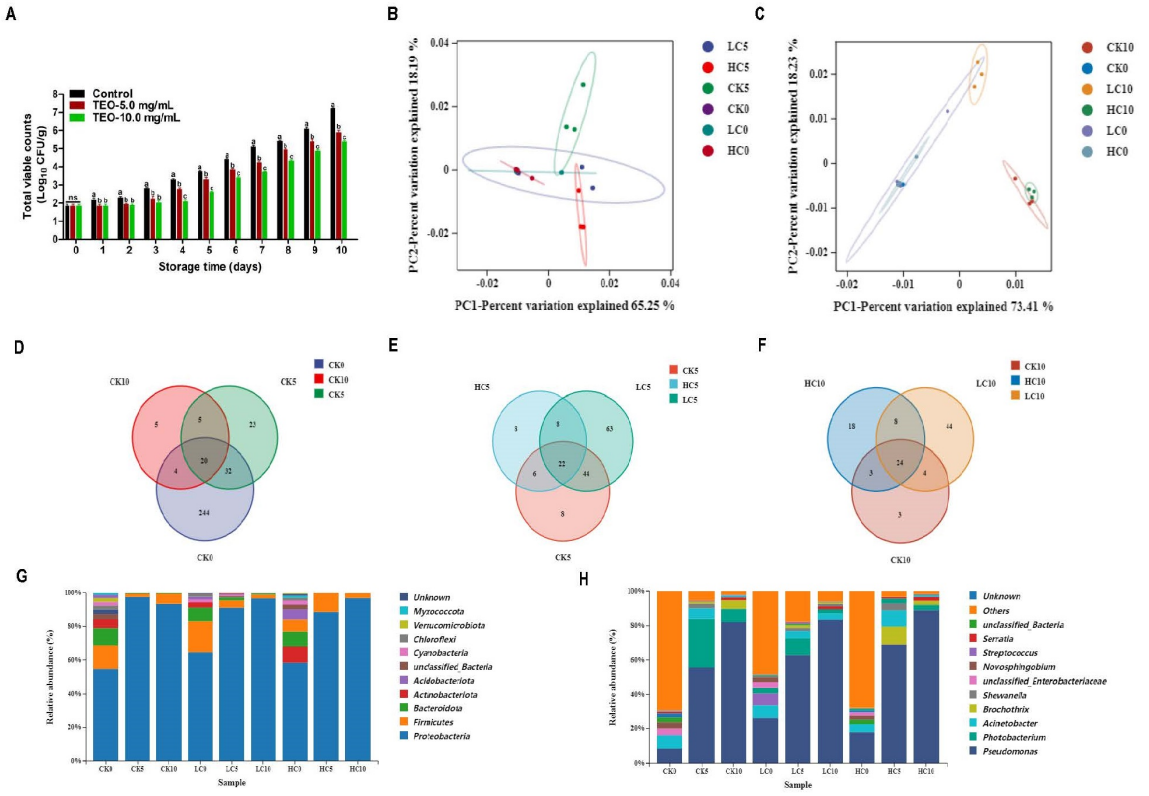


Fig. 2. Total bacterial growth and microbial community analysis of pork treated with thyme essential oil during cold storage. (A) Total bacterial count of pork treated with thyme essential oil during cold storage. Principal component analysis (PCA) was conducted on the bacterial communities present in refrigerated pork samples on days 5 (B) and 10 (C) of the cold storage process. Venn diagrams were used to compare the bacterial communities in untreated and antimicrobial-treated raw pork samples (D–F). The relative abundances (%) of bacterial communities at the phylum level (G) and genus level (H) were analyzed during the refrigeration period. The bacterial taxa with a relative abundance (%) of less than 0.1% were grouped as "other". The control samples, labeled CK0, CK5, and CK10, were stored at 4 °C for 0, 5, and 10 days, respectively. The samples treated with 5.0 mg/mL TEO were denoted as LC0, LC5, and LC10 and were stored at 4 °C for 0, 5, and 10 days. Similarly, samples with a TEO concentration of 10.0 mg/mL were labeled HC0, HC5, and HC10 and stored at 4 °C for 0, 5, and 10 days. All the data are expressed as the means ± standard deviations of three measurements. ns, not significant. Different letters indicate significant differences ($p < 0.05$). TEO, Thyme essential oil.

treated with 5.0 mg/mL TEO showed a slower upward trend within the first 3 days. While the TVC of pork samples treated with 10.0 mg/mL TEO remained relatively stable with almost no increase during this period. From day 5 onwards, all the samples experienced an increase in TVC until the end of the storage period. Notably, the control group displayed more pronounced bacterial growth, whereas the 5.0 mg/mL TEO and 10.0 mg/mL TEO treatment groups effectively inhibited bacterial growth. Significant variations in total viable count (TVC) were observed between the storage commencement and conclusion, with distinct differences observed among the groups subjected to various TEO concentrations ($p < 0.05$). The outcomes of this investigation demonstrated a direct correlation between the quantity of TEO administered and its antibacterial effectiveness. The most noticeable decrease in TVC was observed in the group treated with TEO at 10.0 mg/mL compared to the group treated with TEO at 5.0 mg/mL. From the initial stage of storage to the end of storage, there were significant differences in the TVC between groups treated with different concentrations of TEO ($p < 0.05$). This study demonstrated that the antibacterial activity of TEO was dependent on the dose, with the 10.0 mg/mL TEO group showing the lowest TVC, followed by the 5.0 mg/mL-TEO group. In contrast, the control group without TEO showed a significant increase in TVC. The findings presented in this study highlight the effectiveness of thyme essential oil in inhibiting the growth of microorganisms in chilled pork. This leads to a decrease in spoilage and extends the freshness of the pork by an additional 3 days. The total number of bacterial colonies is a vital parameter for evaluating pork quality and is often influenced by the duration of storage [22]. Thyme essential oil has been found to be effective at prolonging the shelf life of pork through its potential to eliminate various

bacteria. TEO kills a variety of bacteria existed in pork, including many food spoilage-causing bacteria such as *Pseudomonas* and important pathogens such as *Listeria*, *Staphylococcus*, and *Salmonella*. Furthermore, other studies have confirmed that the use of TEO can extend the shelf life of pork and beef [23], which was consistent with our results.

3.2.2 The Result of Microbial Community Analysis

A total of 1,002,124 high-quality 16S rRNA sequences were obtained from all pork samples. Principal component analysis (PCA) revealed distinct clustering of bacterial communities between treatment groups on days 5 and 10 compared to day 0, indicating that TEO treatment altered the microbial community structure during storage (Fig. 2B,C). Venn diagrams (Fig. 2D–F) showed that the control group exhibited a marked decline in both microbial richness and evenness during the later stages of storage, whereas TEO-treated groups maintained significantly higher diversity indices throughout the storage period ($p < 0.05$).

At the phylum level, *Proteobacteria*, *Firmicutes*, and *Bacteroidota* were the dominant taxa across all samples, together accounting for over 78% of total operational taxonomic units (OTUs). With increasing storage time, the relative abundance of *Proteobacteria* increased substantially in both control and TEO-treated groups, while that of *Firmicutes* and *Bacteroidota* decreased (Fig. 2G). However, the TEO-treated groups consistently exhibited lower *Proteobacteria* abundance compared to the control group, whereas the proportions of *Firmicutes* and *Bacteroidota* were relatively lower in TEO-treated samples than in controls.

At the genus level (Fig. 2H), approximately 437 distinct genera were identified across all samples. At the initial storage time (day 0), *Pseudomonas* and *Acinetobacter* were the predominant genera in all experimental groups. During storage, the relative abundance of *Pseudomonas* in the control group increased markedly from 9.20% to 81.23%, whereas *Acinetobacter* decreased from 8.53% to 2.12%. By day 10, *Pseudomonas*, *Photobacterium*, *Serratia*, and *Acinetobacter* collectively accounted for 95.65% of the total microbial community. The relative abundance of *Photobacterium* showed a notable increase from day 0 to day 5 in all groups, while *Serratia* and *Brochothrix* were more abundant in TEO-treated samples compared to controls.

The 16S rRNA sequencing data revealed that TEO treatment not only reduced the total bacterial load but also markedly altered the composition and successional trajectory of the pork-associated microbial community. This observation is consistent with previous reports on other essential oil-based preservatives, such as cinnamon essential oil nanoemulsion and chitosan nanoemulsion [24,25] which similarly suppressed *Proteobacteria* dominance while modulating the abundance of other phyla.

One of the notable findings was the maintenance of higher alpha diversity in TEO-treated groups during later storage stages, whereas control communities rapidly converged toward *Pseudomonas* dominance (Fig. 2D–F). Although increased diversity is frequently linked to ecosystem stability in macroecological contexts [26], its functional significance in food spoilage systems remains inadequately understood. Namely, higher diversity should not be automatically interpreted as evidence of improved microbial stability or preservation. While, several non-mutually exclusive mechanisms could potentially explain the correlation between higher diversity and delayed spoilage observed in this study. First, TEO may act as a moderate selective inhibitor that preferentially suppresses fast-growing Gram-negative spoilage organisms (e.g., *Pseudomonas* and *Photobacterium*), thereby creating vacant ecological niches that allow slower-growing or less proteolytic taxa (e.g., certain *Firmicutes* members) to persist. This community restructuring could contribute to delayed spoilage via intensified resource competition, as a more diverse consortium could more completely utilize limiting substrates such as free amino acids and glucose, which could reduce nutrient availability for residual spoilage populations [27]. However, this hypothesis requires direct metabolomic evidence to confirm substrate depletion dynamics. Second, the increased evenness in TEO-treated communities could potentially interfere with quorum sensing (QS)-mediated coordination of spoilage functions. In low-diversity controls, the high local density of *Pseudomonas* may facilitates rapid accumulation of acyl-homoserine lactone signals, triggering synchronized production of proteases and lipases [28]. The more diverse TEO-treated communities might physically dilute cell-cell contacts or harbor signal-degrading organisms, which could raise the population threshold required for QS activation [29]; nevertheless, this interpretation remains speculative in the context of chilled pork matrices.

At the phylum level, the TEO-mediated suppression of *Proteobacteria*—particularly the marked reduction in *Pseudomonas* dominance—is consistent with the known higher susceptibility of Gram-negative bacteria to phenolic compounds, which is likely attributed to the disruption of outer membrane integrity. The persistence of *Brochothrix* and *Serratia* in TEO-treated samples suggests differential susceptibility among taxa, which may reflect intrinsic differences in cell wall architecture or efflux pump activity. Notably, although these genera are often considered spoilage-associated, their increased relative abundance in the TEO groups did not correlate with accelerated quality deterioration, which suggests that their metabolic contribution to spoilage may be less pronounced than that of *Pseudomonas* under refrigerated conditions [15,24].

Furthermore, the temporal dynamics captured by the sequencing data indicate that TEO delayed the successional shift toward proteolytic genera, rather than simply reducing total biomass. This is supported by the observation that the

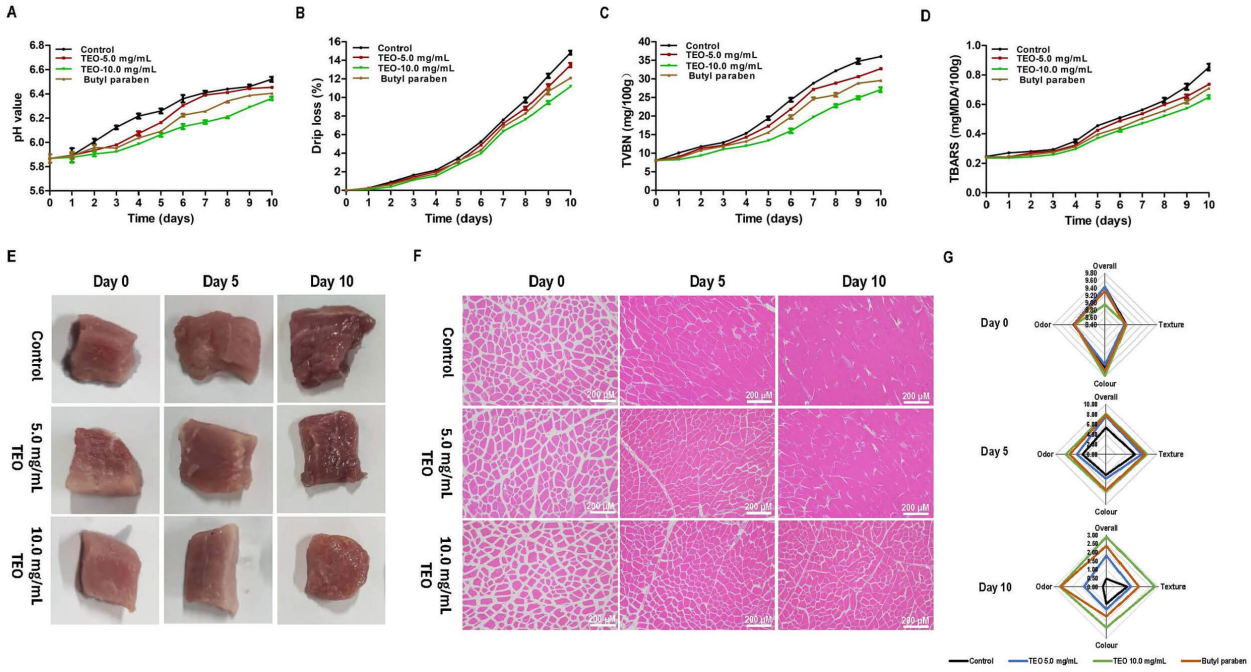


Fig. 3. The quality and freshness of pork preserved with thyme essential oil in cold storage were assessed. (A) pH levels of thyme essential oil-treated pork during storage. (B) Drip loss of pork treated with thyme essential oil during refrigeration. (C) TVB-N content of thyme essential oil-preserved pork during storage. (D) TBARS levels in pork treated with thyme essential oil during refrigeration. (E) Appearance of pork treated with thyme essential oil during cold storage. (F) HE staining of the tissue morphology of pork treated with thyme essential oil during pork storage; scale bars: 200 μm. (G) sensory score radar charts. All the data are expressed as the means ± standard deviations of three measurements. TVB-N, total volatile basic nitrogen; TBARS, thiobarbituric acid reactive substances; HE, Hematoxylin and Eosin stain.

5.0 and 10.0 mg/mL TEO groups, despite having comparable total viable counts at day 10, exhibited divergent physicochemical profiles (lower TVB-N and TBARS at the higher concentration), suggesting that community composition—rather than absolute cell numbers—could be a more relevant influencing factor of spoilage outcomes.

3.2.3 pH and Drip Loss Measurement

As demonstrated in Fig. 3A, the application of TEO and butylparaben effectively prevented the pH of the pork from increasing during storage compared with that of the control group. Additionally, Fig. 3B illustrates that the use of TEO at a concentration of 10.0 mg/mL led to minimal drip loss during storage, indicating its high efficacy in preserving the quality of raw and chilled pork products. During refrigeration, the pH of the pork may decrease due to the inhibition of bacterial growth, possibly caused by TEO. This reduction can be attributed to the reduced production of volatile basic nitrogen molecules such as amines [30]. These findings highlight the utility of TEO in this regard.

3.2.4 Changes in TVB-N and TBARS

The freshness of pork can be evaluated by the concentration of TVB-N, which is predominantly generated by spoilage bacteria-mediated proteolysis [31]. As illustrated in Fig. 3C, the TVB-N concentration decreased in a dose-dependent manner in all experimental groups compared with the control group. On day 10, the TEO treatment groups (5.0 and 10.0 mg/mL) had TVB-N values of 32.5 and 27.5 mg/100 g, respectively. Similarly, the application of butylparaben also suppressed TVB-N production, yielding a concentration of 29.7 mg/100 g. The outstanding antibacterial characteristics of TEO, similar to those of biopreservatives such as cinnamon essential oil [25] and *Cornus mas* L. leaf extracts [32,33], could explain these findings. Pork oxidation during storage is assessed by measuring TBARS level, as indicated by the measurement of malondialdehyde (MDA) concentrations. Fig. 3D shows that the TBARS values of each experimental group increased over the storage period. Notably, the presence of 10.0 mg/mL TEO effectively inhibited the increase in MDA, with a measured MDA concentration of 0.64 mg/100 g on day 10. Furthermore, butylparaben also inhibited TBARS, resulting in an MDA concentration of 0.72 mg/100 g on day 10. Notably, when exposed to 5.0 mg/mL TEO, the concentration in the TEO-treated sample was 0.75 mg/100 g, while the concentration in the control group after 10 days was approximately 0.87 mg/100 g. The TBARS concentration is an indicator commonly employed for assessing lipid oxidation levels in fresh pork, where a higher TBARS value

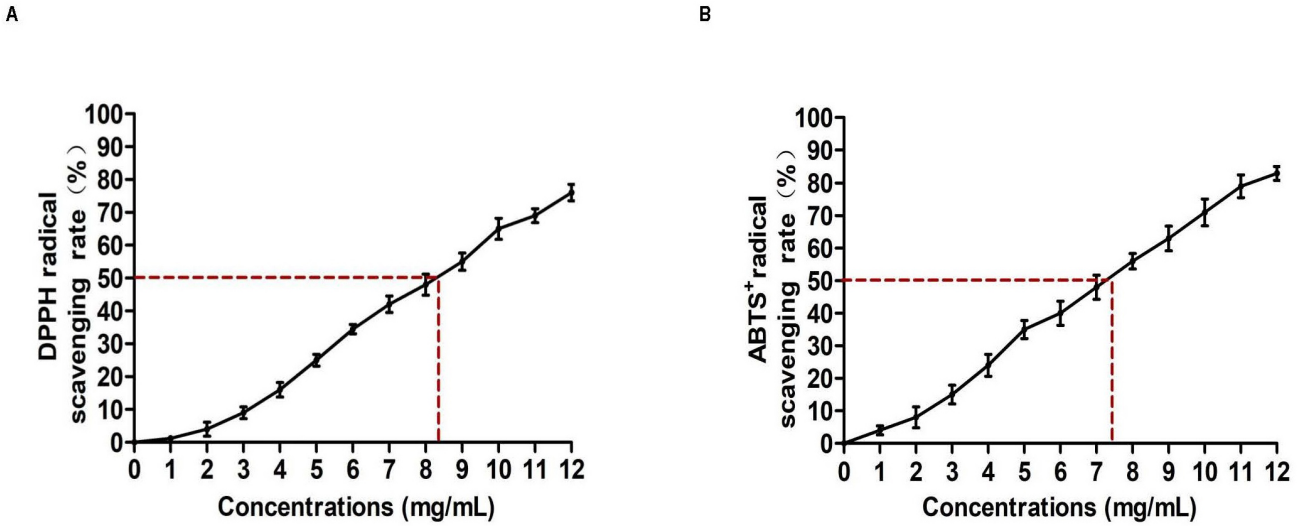


Fig. 4. Antioxidant activity of TEO. (A) 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. (B) 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^+$) radical scavenging assay. All the data are expressed as the means ± standard deviations of three measurements.

indicates more severe oxidation [34]. The results indicated that the application of TEO has a favorable influence on the preservation of refrigerated meat. This observation remains in agreement with the *in vitro* results obtained for oregano oil, which revealed its antibacterial and antioxidant properties. Therefore, the results of the TBARS test can be attributed to the antioxidant function of TEO, indicating that thyme essential oil has the potential to inhibit TBARS production in pork and effectively resist lipid oxidation, aligning with the findings of *in vitro* evaluations.

3.2.5 Sensory Quality

Sensory evaluation is used to subjectively assess the overall quality of a food product over time. According to Table 2, the sensory evaluation of the pork in the control group significantly decreased as the storage period increased. The sensory score decreased to less than 5 points on the 6th day. However, on day 6, the sensory scores of the 10 mg/mL TEO-treated group decreased only slightly ($p < 0.05$), and the difference was statistically significant compared with that of the control group. According to the prescribed sensory evaluation standards, the TEO treatment groups at different concentrations (5.0 and 10.0 mg/mL) all reached acceptable sensory values (>6) on days 5 and 6, respectively. Furthermore, the control group displayed noticeable changes in muscle cell efficiency and distinct morphological changes, as observed through the use of HE staining. Cell morphology observation revealed that the cell morphology of cells in the thyme essential oil group changed at concentrations of 5.0 mg/mL and 10.0 mg/mL (Fig. 3E,F). The tissue morphology of the 10.0 mg/mL thyme essential oil treatment group remained basically unchanged, which was consistent with the sensory results (Fig. 3G). The sensory score radar chart is shown in Fig. 3G at 0, 5 and 10 days, respectively, which is consistent with the morphology observation.

Table 2. Sensory scores of pork samples treated with control, 5 and 10 mg/mL thyme essential oil (TEO), and butyl paraben during 0–10 days of storage.

Days	Control	TEO		Butyl paraben
		5.0 mg/mL	10.0 mg/mL	
0	9.33 ± 0.26	9.33 ± 0.28	9.24 ± 0.13	9.22 ± 0.20
1	9.32 ± 0.13	9.30 ± 0.17	9.22 ± 0.12	9.20 ± 0.14
2	8.69 ± 0.21	9.18 ± 0.18	9.21 ± 0.15	9.24 ± 0.18
3	8.24 ± 0.29	9.01 ± 0.16	9.25 ± 0.14	9.16 ± 0.25
4	7.23 ± 0.26	8.25 ± 0.18	8.79 ± 0.25	8.53 ± 0.21
5	5.23 ± 0.23	6.54 ± 0.19	8.14 ± 0.17	7.46 ± 0.23
6	4.25 ± 0.27	5.32 ± 0.31	7.34 ± 0.26	6.41 ± 0.28
7	3.26 ± 0.30	4.28 ± 0.27	5.34 ± 0.19	4.66 ± 0.23
8	2.19 ± 0.25	3.84 ± 0.17	5.02 ± 0.26	4.12 ± 0.32
9	1.72 ± 0.21	2.87 ± 0.23	4.35 ± 0.31	3.76 ± 0.29
10	0.50 ± 0.22	1.61 ± 0.26	2.94 ± 0.17	2.16 ± 0.31

Control samples were treated with sterile saline solution; TEO groups received 5 or 10 mg/mL TEO; butyl paraben was applied at a concentration of 0.05% (w/v).

3.2.6 Antioxidant Activity of TEO

Fig. 4A,B shows the analysis of the antioxidant activity of TEO, specifically its ability to scavenge DPPH and $ABTS^+$ anionic radicals. The measured IC50 values were 8.20 and 7.50 mg/mL, respectively. The findings demonstrated that the antioxidant effects of TEO exhibited a dose-dependent relationship. Additionally, it is important to note that the deterioration in pork quality can be influenced by

microbial growth and nutrient oxidation. In contrast to the results of prior investigations, essential oils, such as rosemary EO, *Litsea pungens* EO [35], and *Taraxacum officinale* EO [36], which scavenge free radicals, were also found to support the prevention of lipid oxidation. Numerous studies have demonstrated the potent antioxidant properties of thyme essential oil [37,38]. The IC50 values obtained in our study align with the findings of Hussain et al. [39], who reported that *T. serpyllum* EO exhibited the highest antioxidant capacity. The disparities between our results and theirs can be attributed to variations in the analytical methods employed. Additionally, the compositions of the two oils differ, leading to distinct abilities to participate in these mechanisms. The most important factor in evaluating the antioxidant activity of an oil is its phenolic content. The effectiveness of oils in preventing diseases caused by oxidative stress is mainly attributed to their ability to scavenge free radicals and bind metals. Therefore, these results indicate that TEO, as a plant essential oil, can act as an antioxidant and help maintain pork quality.

4. Conclusion

This study demonstrated that thyme essential oil (TEO) possesses potent, potential multiple targets antibacterial activity and notable antioxidant capacity, confirming its potential as an effective natural preservative for refrigerated pork. TEO exhibited broad-spectrum antibacterial effects with MICs of 0.5–2.5 mg/mL, and mechanistic investigations revealed that it disrupts bacterial membrane integrity and causes leakage of intracellular contents. DNA gel retardation assays further showed that TEO components can bind to purified genomic DNA *in vitro*, suggesting a possible secondary mechanism. These combined effects ultimately lead to bacterial cell death. In refrigerated pork, the 10.0 mg/mL TEO treatment was most effective, inhibiting microbial growth and modulating the bacterial community composition by reducing the dominance of spoilage-associated genera such as *Pseudomonas*, while maintaining higher species evenness throughout storage. These antimicrobial effects, combined with the oil's intrinsic antioxidant activity, delayed quality deterioration, as reflected in the maintenance of lower pH and drip loss, reduced TVB-N and TBARS levels, and the preservation of tissue integrity and superior sensory scores throughout 10 days of storage at 4 °C. These effects are attributed to the synergistic action of membrane-active and DNA-binding properties and free radical scavenging, likely primarily driven by phenolic compounds such as thymol. Although the precise contributions of individual constituents remain to be determined without a full compositional analysis. The findings demonstrate TEO's potential as a clean-label, natural alternative to chemical preservatives, supporting its use in the development of functional and sustainable meat products.

5. Limitations

Although TEO has been studied for meat preservation, a few points deserve mention regarding the present work. First, TEO is a complex mixture of bioactive compounds (e.g., thymol and cinnamyl alcohol), yet the possible interactions among these constituents require further investigation. Second, the observed antimicrobial effects are clear, but the detailed intracellular targets still need to be explored in greater depth. Meanwhile, the proposed ecological hypotheses (resource competition, quorum sensing interference) await experimental validation using approaches such as metatranscriptomics or synthetic community models. Nevertheless, aside from these aspects, the sequencing results do provide meaningful insights, revealing the taxonomic selectivity and temporal dynamics of TEO action—information that cannot be obtained through conventional plating. We consider these as open questions for future studies, and they do not affect the overall conclusions of this work.

Availability of Data and Materials

The data that support the findings of this study are not publicly available due to the protection of patent-related intellectual property and ongoing technology development. Data are available from the corresponding author upon reasonable request, subject to institutional approval and confidentiality obligations.

Author Contributions

ZS: Writing and designed this study. XY: Plotted data graphs. YL: Funded this research and desigined this study. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Conflicts of Interest

The authors declare no conflicts of interest.

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