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Antilisterial activity of *Thymus vulgaris* essential oil: *In vitro*, *in situ*, and *in silico* investigations

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ABSTRACT

Listeria monocytogenes is a major foodborne pathogen that significantly threatens public health and food safety. While *Thymus vulgaris* essential oil (TV-EO) is widely recognized for its potent antibacterial activity, its specific effects against *L. monocytogenes* remain unexplored. This study aimed to assess the antilisterial activity of TV-EO using *in vitro*, *in situ*, and *in silico* approaches. The *in vitro* assessment included disc diffusion method, determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), biofilm inhibition assay, and predictive modeling to assess *L. monocytogenes* reduction in the presence of TV-EO at 10 °C and 20 °C. *In situ* approach evaluated the inhibitory effect of TV-EO on *L. monocytogenes* in minced poultry meat stored at 4 °C. Finally, *in silico* approach, based on molecular docking, was employed to evaluate the binding affinity of major TV-EO components for β-ketoacyl-ACP synthase II and chorismate synthase, key proteins involved in fatty acid biosynthesis and biofilm formation, respectively. Our finding revealed that TV-EO exhibited strong *in vitro* antilisterial activity, with inhibitory zones ranging from 51.00 ± 1.00 mm to 55.67 ± 1.15 mm, a MIC value of 0.125 %, and a MBC value of 0.25 %, indicating its bactericidal effect. TV-EO at 0.125 % demonstrated a high capacity to inhibit and eradicate the biofilm, with 100 ± 0.00 % and 91.33 ± 1.23 %, respectively. Predictive modeling, based on the combination of TV-EO and ζ values, revealed that *L. monocytogenes* inactivation was more pronounced at low temperature. Furthermore, the *in-situ* approach showed a significant reduction of *L. monocytogenes* amount, with decreases of 1.068 ± 0.132 log cfu/g, 0.671 ± 0.091 log cfu/g, and 0.317 ± 0.029 log cfu/g at TV-EO concentrations of 1 %, 0.5 %, and 0.25 %, respectively ($p < 0.05$). *In silico* analysis indicated that TV-EO components, particularly carvacrol, exhibited high affinity for β-ketoacyl-ACP synthase II and chorismate cynthase, suggesting strong antilisterial and ani-biofilm activity. These findings highlight the antilisterial efficacy of TV-EO, demonstrating its potential as a natural alternative to conventional preservatives for enhancing food preservation and safety.

1. Introduction

The increase in the global population has led to a higher demand for food, particularly fast food and ready-to-eat products. Consequently, this growing demand poses a greater risk of pathogens transmission to consumers, resulting in severe cases of foodborne illnesses [1]. According to the World Health Organization (WHO), an estimated 600 million individuals fall ill each year due to consuming contaminated

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food, resulting in 420,000 fatalities annually and a loss of 33 million healthy life years [2]. In the United States, approximately 9.4 million cases of foodborne illness occur annually, resulting in 55,961 hospitalizations and 1351 deaths [3]. In 2022, 5763 foodborne outbreaks were reported in the European Union and United Kingdom, leading to 48,605 cases of illness, 2783 hospitalizations, and 64 deaths [4]. Notably, diarrheal diseases are the prevalent illnesses associated with contaminated food, affecting 550 million people annually and causing 230,000 deaths [5]. Therefore, preventing the transmission of foodborne pathogens along the food chain is essential to reducing human exposure to these pathogens and, consequently, lowering the incidence of foodborne diseases.

Listeria monocytogenes is a Gram-positive bacterium widely distributed in nature and found in soil, water, and farming environments [6–8]. Foods, particularly those of animal origin and ready-to-eat products, can become contaminated at various stages of food processing chain. During distribution and storage, especially under refrigeration temperatures, *L. monocytogenes* can proliferate to hazardous levels [9]. As result, numerous studies have reported the presence of *L. monocytogenes* in various food products, particularly in uncooked or undercooked foods [10,11]. Additionally, according to recent studies, dairy, fresh produce, and meat-poultry are the primary source of *L. monocytogenes* infections (listeriosis) in the US [12]. Although listeriosis is relatively rare, it can lead to severe infections in both humans and animals, with mortality rates ranging from 20 % to 30 % [13]. Recently, the continuous rise of antimicrobial resistance across various sectors, including veterinary medicine, human healthcare, and food production, has made combatting antimicrobial resistance in foodborne pathogens increasingly challenging [14]. Addressing this issue requires the development of new, sustainable, and more effective control strategies [15–18].

In food processing chain, *L. monocytogenes* can withstand various environmental stressors, including low temperatures, acidic pH, high salt levels, and desiccation, enabling its persistence and survival in food processing environments such as refrigeration units, processing machinery, and packaging materials [19–21]. Additionally, *L. monocytogenes* has been shown to exhibit resistance to disinfectants [22]. Notably, resistance to environmental stressors and disinfectants may contribute to the development of antimicrobial resistance [15,23]. Furthermore, biofilm formation further enhances the resistance of *L. monocytogenes* to cleaning and sanitation procedures [21,24]. This persistence underscores the critical need for stringent food safety measures and continuous monitoring to minimize the risk of *L. monocytogenes* contamination and ensure the safety of food products.

Essential oils (EOs) are volatile secondary metabolites extracted from plant materials and are characterized by their distinct aromatic properties. They play a crucial role in plant defense and exhibit various beneficial properties, including antioxidant, antimicrobial, antiviral, anti-inflammatory activities, among others [25,26]. To date, numerous studies have showcased the antimicrobial activity of EOs against a wide range of spoilage and pathogenic microorganisms, including Gram-negative and Gram-positive bacteria, molds, yeasts, and parasites [27–31]. Notably, EOs from aromatic plants are being utilized as safe and effective alternative to synthetic preservatives for combating pathogenic bacteria in food products [25,32–34].

Thymus vulgaris essential oil (TV-EO) offers a compelling range of benefits as an alternative food preservative. First and foremost, its potent antimicrobial properties effectively inhibit the growth of various harmful bacteria, fungi, yeast, and parasites that contribute to food spoilage and contamination [27,35]. Additionally, TV-EO exhibits antioxidant activity, which helps prevent oxidative deterioration of food products, thereby extending their shelf life [36,37]. Moreover, TV-EO is considered relatively safe for consumption when used in appropriate concentrations, making it a preferable alternative to synthetic preservatives, which may pose potential health risks [36]. Furthermore, its pleasant aroma and flavor can enhance the sensory appeal of preserved foods, adding an extra dimension to culinary applications [38]. Overall, the use of TV-EO as a sustainable food preservative not only enhances food safety and shelf life but also aligns with the growing consumer demand for natural and minimally processed ingredients in food products.

This study aims to evaluate the antilisterial activity of TV-EO using *in vitro*, *in silico*, and *in situ* approaches. The *in vitro* experiments assess the effect of TV-EO on *L. monocytogenes* strains by determining inhibition zones, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), biofilm formation, and effect on growth kinetics. The *in situ* approach evaluates TV-EO's antilisterial efficacy in poultry minced meat stored at 4 °C for 13 days. While the *in silico* approach, utilizing molecular docking, aims to elucidate the antilisterial and antibiofilm mechanisms of major TV-EO components.

2. Material and method

2.1. Essential oil and bacterial strains

The essential oil was extracted from *T. vulgaris* leaves collected from the mountainous region of Ifrane, Morocco, using hydrodistillation in a Clevenger-type apparatus (IsoLab Laborgäte GmbH, Wertheim, Germany). The chemical composition of TV-EO was analyzed using gas chromatography coupled to mass spectrometry (GC–MS), revealing thymol (38.68 %) as the predominant component, followed by p-cymene (15.66 %), carvacrol (14.89), and γ-terpinene (13.31 %), among others [37]. The L. *monocytogenes* strains used in this study included wild type strains isolated from different food samples, as well as reference strains from the American Type Culture Collection (Table 1). To conduct experiments, a loopful of each *L. monocytogenes* strain was taken from storage culture (−80 °C), sub-cultured on Tryptone Soy Yeast Extract Agar (TSYEA, Biolife, Milan, Italy) and incubated at 37 °C for 24 h–48 h.

2.2. In vitro inhibition of L. monocytogenes by TV-EO

The *in vitro* antilisterial activity of TV-EO was assessed using disc diffusion method to determine the inhibitory zone and broth microdilution method to determine MIC and MBC, following a previously published protocol [30]. Briefly, bacterial suspensions equivalent to 0.5 McFarland (about 10^6 CFU/mL) were prepared using sterile physiological water (0.9 % NaCl; 0.9 g of NaCl in 100 mL of distilled water), and swabbed onto Mueller-Hinton Agar (MHA, Biolife, Milan, Italy) plates. The plates were then allowed to dry at room temperature for 15 min. Afterward, sterile paper discs (6 mm in diameter; Biolife, Milan, Italy)

Table 1
The MIC and MBC in % (v/v) of TV-EO against *L. monocytogenes* strains.

ID strains	Information[a]	Serotype	Origin	MIC	MBC	MBC/MIC
L1	ListME222	–	Wild type-ice cream	0.125 %	0.25 %	2
L2	ATCC 13932	4b	Human	0.125 %	0.25 %	2
L3	ListME212	–	Wild type-meat product	0.125 %	0.25 %	2
L4	ATCC 7644	1/2c	Human	0.125 %	0.25 %	2
L5	ATCC 19111	½	Poultry	0.125 %	0.25 %	2
L6	ListME1	–	Wild type-smoked Salmon	0.125 %	0.25 %	2
L7	ListME9	–	Wild type-fresh salmon	0.125 %	0.25 %	2
L8	ListME13	–	Wild type-smoked Salmon	0.125 %	0.25 %	2

[a] ATCC: American Type Culture Collection.

were impregnated with 10 μL of TV-EO and placed on the inoculated plates. A disc soaked with 10 μL of 5 % Tween 80 (Sigma-Aldrich, USA) served as a negative control, while Cefotaxime (CTX, 30 μg) was used as a standard reference. After incubation at 37 °C for 24 h, the inhibitory zones of TV-EO against each *L. monocytogenes* strain were measured in millimeter, including the diameter of the disc. All the experiments were conducted in triplicates, and results were expressed as means ± Standard Deviation of three replicates.

The MIC values were determined using the broth dilution method. Briefly, serial dilutions of TV-EO (ranging from 4 % to 0.0625 %; v/v) were prepared in microtubes containing 100 μL of Tryptone Soya Yeast Extract Broth (TSYEB) (Biolife, Milan, Italy) supplemented with 5 % Tween 80 (Sigma-Aldrich, USA). Subsequently, 4 μL of bacterial suspensions (equivalent to 0.5 McFarland) were added to each microtube, mixed thoroughly, and incubation at 37 °C for 24 h. Microtubes containing only TSYEB served as negative controls, while microtubes containing TSYEB inoculated with bacterial suspensions served as positive controls. After incubation, MIC values were determined as the lowest concentration of TV-EO that showed no visible bacterial growth. However, MBC values were determined by sub-culturing 5 μL from microtubes without visible growth onto TSYEA plates, followed by incubation at 37 °C for 24h. The lowest concentration that showed no bacterial growth on TSYEA plates was considered as the MBC.

2.3. In situ inhibition of L. monocytogenes by TV-EO

Poultry minced meat, previously contaminated with *L. monocytogenes* (3.0 log cfu/g), was divided into five batches. One batch was preserved as a negative control (without TV-EO treatment), while the remaining four batches were treated with different concentrations of TV-EO (0.125 %, 0.25 %, 0.5 %, 1 %; v/w). All samples were stored at 4 °C and periodically examined for *L. monocytogenes* counts. To achieve this, 25 g of each sample was mixed with 225 mL of sterile peptone water, followed by the preparation of decimal dilutions. The enumeration of *L. monocytogenes* was performed by plating 0.1 mL of each dilution on Agar Listeria according to Ottaviani & Agosti (ALOA; Biolife, Milan, Italy), followed by incubation at 37 °C for 24 h. Colonies exhibiting a blue to blue-green color surrounded by opaque halo were counted as *L. monocytogenes*, and the results were presented in cfu/g.

2.4. Predictive modeling of L. monocytogenes growth and inactivation kinetics

To characterize the effect of TV-EO on the growth and inactivation kinetics of *L. monocytogenes*, a predictive microbiology model previously employed for *Salmonella enterica* in the presence of TV-EO was used [37]. In detail, the model introduces a variable that simulates the inhibiting effect of TV-EO into the primary model instead of the secondary one. This approach avoids interference with the growth rate calculation, allowing the use of a generic secondary model based on the main environmental variables. The model is based on the set of differential equations of Baranyi and Roberts' model [39], where the bacterial concentration $\boldsymbol{N}$ at time $\boldsymbol{t}$ is generically expressed as follows:

$$\boldsymbol{dN/dt} = \boldsymbol{\mu_{max}} * \boldsymbol{N} * [\boldsymbol{Q}/(1+\mathrm{Q})] * [1-(\boldsymbol{N}/\boldsymbol{N_{max}})] \quad (1)$$

$$\boldsymbol{dQ/dt} = \boldsymbol{\mu_{max}} * \boldsymbol{Q} \quad (2)$$

Here $\boldsymbol{\mu_{max}}$ is the maximum specific growth rate and $\boldsymbol{N_{max}}$ the theoretically maximum population densities of the bacterial population; $\boldsymbol{Q}$ represents the physiological state of the species and allows to express the lag phase duration.

According to Ed-Dra et al. [37] the effect of TV-EO on the bacterial dynamic was simulated introducing the term $\boldsymbol{\zeta}$ into equation (2), as expressed in equation (3).

$$\boldsymbol{dN/dt} = \boldsymbol{\mu_{max}} * \boldsymbol{N} * [\boldsymbol{Q}/(1+\mathrm{Q})] * [1-(\boldsymbol{N}/\boldsymbol{N_{max}})] * \boldsymbol{\zeta} \quad (3)$$

with: $\boldsymbol{\zeta = 1}$ in absence of TV-EO; $\boldsymbol{\zeta < 1}$ and $\boldsymbol{\zeta > 0}$ when a growth of *L. monocytogenes* is observed; $\boldsymbol{\zeta < 0}$ when a decrease of concentration of *L. monocytogenes* is observed.

Equations (2) and (3) were used to model the kinetics of *L. monocytogenes* in both *in vitro* and *in situ* tests. These equations were numerically solved using the secondary model proposed by Le Marc et al. [40] to calculate $\boldsymbol{\mu_{max}}$ under the specific environmental conditions of each test. The initial Q value ($\boldsymbol{Q_0}$) was obtained using the Solver function in Excel (Microsoft Corporation, New York, USA) by fitting the predicted kinetics to the observed ones, obtained at 0 % of TV-EO. Likewise, the $\boldsymbol{N_{max}}$ values were considered as the maximum population density observed in tests without TV-EO. The obtained $\boldsymbol{Q_0}$ and $\boldsymbol{N_{max}}$ and the secondary model for $\boldsymbol{\mu_{max}}$, were used to solve equation (3) and to calculate $\boldsymbol{\zeta}$ values for each test (*in vitro* and *in situ*) in the presence of TV-EO, by fitting (with the Solver function of Excel) the predicted kinetics to the observed ones, obtained in presence of TV-EO. All fitting procedures were performed by minimizing the Root Mean Squared Error (RMSE) between observed and predicted data.

2.5. Anti-biofilm effect of TV-EO

2.5.1. Biofilm inhibition assay

The inhibition effect of TV-EO at MIC and MIC/2 concentrations against *L. monocytogenes* ATCC 13932 biofilm formation was performed using crystal violet staining assay [31,41]. Briefly, 0.5 mL of TSYEB containing TV-EO (at MIC and MIC/2 concentrations) and DMSO (Loba Chemie, India) at a final concentration of 5 % was prepared in a sterile 24-well microplate (Labbox, Spain). To each well, 100 μL of bacterial suspension (equivalent to 10^6 CFU/mL) was added. A well containing TSYEB-DMSO (5 %) with bacterial suspension (without TV-EO) served as the positive control, while a well containing TSYEB with DMSO (5 %) (without bacterial suspension) served as negative control.

The microplate was incubated at 37 °C for 24 h without shaking to allow the bacterial cells to adhere to the surface. After incubation, the contents of each well were discarded, and the wells were rinsed three times with sterile phosphate buffered saline (PBS) to remove planktonic cells, including loosely attached and non-adherent cells. The plate was then air-dried and subsequently dried by heating at 60 °C for 45 min. Next, the wells were stained with 600 μL of 1 % crystal violet (Sigma-Aldrich, USA) and left at room temperature for 15 min. Following staining, the wells were rinsed three times with PBS to remove unabsorbed stain and destained with 1 mL of ethanol (VWR Chemicals, China). The contents of each well were transferred to a cuvette, and absorbance was measured at 590 nm using a spectrophotometer (SELECTA, Spain).

Each experiment was conducted in triplicate, and the mean absorbance of the samples was calculated. The percentage inhibition was then determined according to the following formula [42]:

$$\%\ \text{Inhibition} = \frac{OD_{positive\ control} - OD_{sample}}{OD_{positive\ control}} \quad (4)$$

2.5.2. Biofilm eradication assay

To evaluate the eradication capacity of TV-EO against the biofilm formed by *L. monocytogenes*, biofilm formation was first carried out in sterile 24-wells microplate (Labbox, Spain). The formed biofilm was then treated with TV-EO at MIC and MIC/2 concentrations [31].

To allow biofilm formation, 0.5 mL of TSYEB containing DMSO (at finale concentration of 5 %) and 100 μL of bacterial suspension (10^6 CFU/mL) were prepared in sterile 24-well microplates and incubated without shaking at 37 °C for 24 h. A well containing only TSYEB and DMSO was used as the negative control. After incubation, the bacterial suspensions (planktonic cells) were carefully removed, and wells were rinsed three times with PBS. Subsequently, 0.5 mL TSYEB-DMSO containing TV-EO (at MIC and MIC/2 concentrations) was added to each well and incubated without shaking at 37 °C for 24 h. A well containing

only TSYEB-DMSO (without TV-EO) served as the positive control. After incubation, the wells were rinsed three times with PBS and stained with crystal violet as described in section 2.5.1. After staining, 1 mL of ethanol was added to each well, and the absorbance was measured at 590 nm using a spectrophotometer (SELECTA, Spain). Each experiment was conducted in triplicates, and the percentage of biofilm eradication was determined according to the formula provided above (4).

2.6. In silico assessment

To better understand the antibacterial and anti-biofilm potential of TV-EO against *L. monocytogenes*, molecular docking was used to assess the effect of the major components identified in TV-EO on chorismate synthase, an essential protein involved in the biofilm pathway [43,44], and β-ketoacyl-ACP synthase II, a protein involved in fatty acid biosynthesis [45].

The crystal structures of chorismate synthase (PDB ID: 1Q1L) and β-ketoacyl-ACP synthase II (PDB ID: 5SXO) were retrieved from the Protein Data Bank (PDB) (available online: www.rcsb.org) [46]. Each structure was individually prepared by removing water molecules and carefully adding polar hydrogens and Kollman charges using AutoDockTools (ADT; version 1.5.7) [46]. For molecular docking studies, a grid with a spacing of 0.375 Å and dimensions of 40 × 40 × 40 was defined, centered on the x, y, and z coordinates to cover both the active sites and peripheral regions of the proteins. The prepared macromolecules were then saved in pdb format for subsequent molecular docking analysis [47].

Additionally, to prepare the ligands, the major components identified in TV-EO (*p*-cymene, γ-terpinene, thymol, and carvacrol), along with platensimycin (CID: 6857724) and juglone (CID: 3806) as standard inhibitors, were retrieved in 3D SDF format from the PubChem database (available online: https://pubchem.ncbi.nlm.nih.gov/). These compounds were evaluated for their potential to inhibit chorismate synthase and β-ketoacyl-ACP synthase II through molecular docking studies. Using PyMOL (version 2.5.3), the ligands were first converted to pdb format, then further processed into pdbqt format via AutoDockTools (ADT; version 1.5.7, The Scripps Research Institute) [48].

The molecular interactions between ligands and receptors, along with their binding affinities relative to the standard inhibitor, were analyzed using BIOVIA Discovery Studio Visualizer (21.1.0.0), which also facilitated graphical representation [47].

2.7. Statistical analysis of data

The experiments were conducted in triplicates and the results were presented as means ± standard Deviation (S.D.). The difference between groups was statistically analyzed using Student's t-test and ANOVA. A statistically significant difference was considered for $p < 0.05$. All the statistical analyses were performed using Microsoft Excel (New York, USA) and GraphPad Prism version 9 software (GraphPad, San Diego, CA, USA)

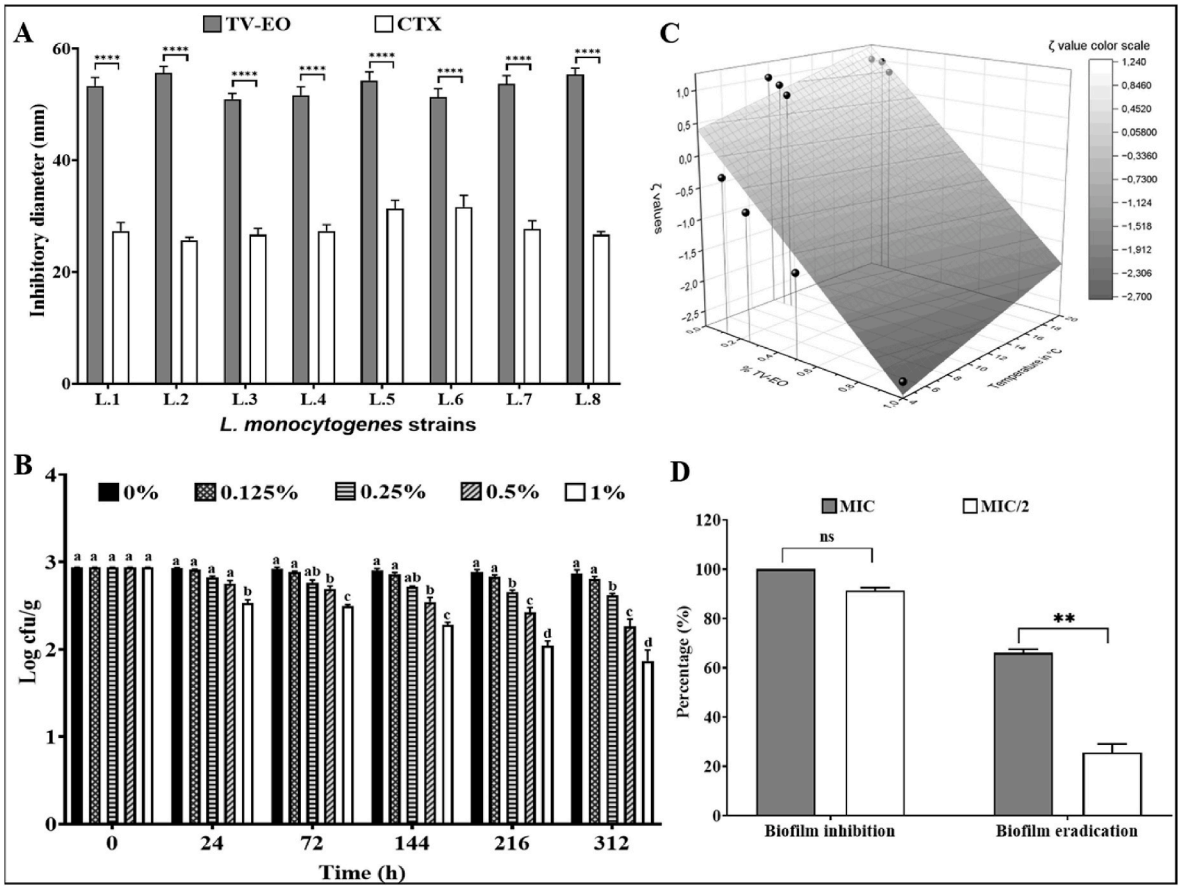

Fig. 1. Antilisterial and antibiofilm activity of TV-EO. (**A**) Inhibition diameters (mm) of TV-EO against *L. monocytogenes* strains. (**B**) Inactivation dynamics of *L. monocytogenes* (log cfu/g) during the storage of poultry minced meat treated with different concentrations of TV-EO; Different letters in the same group indicate significant differences ($p < 0.05$). (**C**) graphical representation of multiple linear regression with "%TV-EO" and "T in °C" as independent variables and "ζ" as dependent variable ($R^2 = 0.9334$). (**D**) Capacity of TV-EO to inhibit and eradicate the formation of biofilm by *L. monocytogenes* at MIC and MIC/2 concentrations.

3. Results

3.1. In vitro antilisterial activity of TV-EO

The antibacterial activity of TV-EO against *L. monocytogenes* strains was assessed using disc diffusion and broth microdilution methods. The results obtained from disc diffusion method showed that the inhibitory diameters vary between 51.00 ± 1.0 mm and 55.67 ± 1.15 mm (Fig. 1A). It is noteworthy that the antibacterial activity of TV-EO was significantly higher than that of the reference standard (Cefotaxime, 30 µg) ($p < 0.0001$). Similarly, the broth dilution assay showed that TV-EO exhibited strong antilisterial activity, with MIC values of 0.125 % (v/v) and MBC values of 0.25 % (v/v), indicating its bactericidal effect (MBC/MIC<4) (Table 1).

3.2. In situ antilisterial activity of TV-EO

The antilisterial effect of TV-EO in poultry minced meat was evaluated, and the results are presented in Fig. 1B. Our findings indicate that TV-EO significantly reduced the *L. monocytogenes* load after 24 h when treated with a concentration of 1 %, after 72 h with a concentration of 0.5 %, and after 216 h with a concentration of 0.25 % ($P < 0.05$). No significant difference was observed for samples treated with 0.125 % of TV-EO ($P < 0.05$). Additionally, our results revealed that after an incubation time of 312 h, there was a reduction of 1.068 ± 0.132 log cfu/g for 1 % concentration, 0.671 ± 0.091 log cfu/g for the 0.5 %

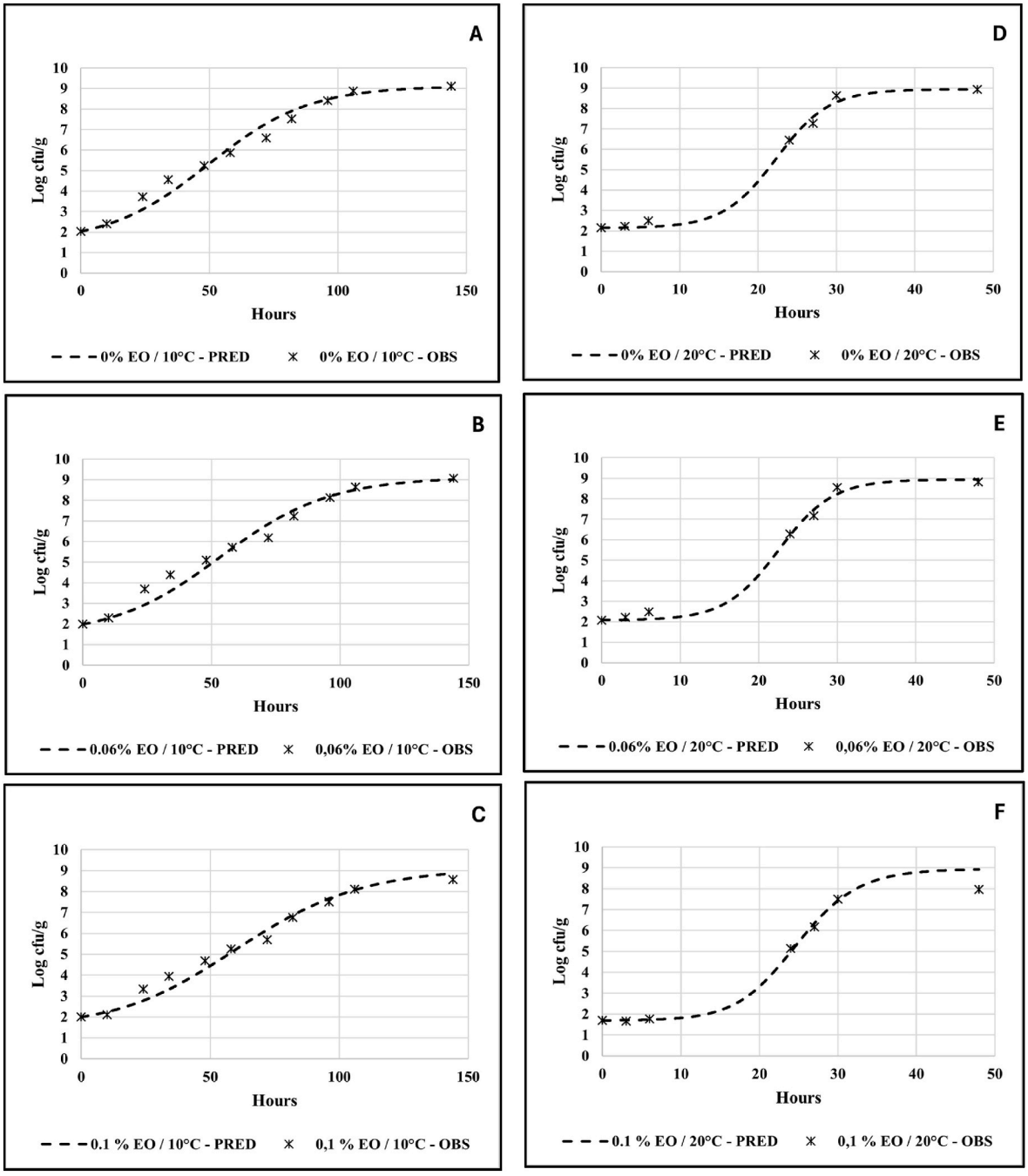

Fig. 2. Observed and predicted behaviour of *L. monocytogenes* for *in vitro* tests at 10 °C (panel A–C) and 20 °C (panel D–F), with 0 % TV-EO (panel A and D), 0.06 % TV-EO (panel B and E), 0.1 % TV-EO (panel C and F).

concentration, 0.317 ± 0.029 log cfu/g for the 0.25 % concentration, and 0.129 ± 0.022 log cfu/g for the 0.125 % concentration. The untreated samples (0 %) maintained approximately stable amounts, with a reduction of 0.063 ± 0.030 log cfu/g.

3.3. Prediction modeling

The predicted dynamics of *L. monocytogenes* under increasing concentrations of TV-EO for *in vitro* and *in situ* tests are reported in Figs. 2 and 3, respectively. The RMSE for each test and the main parameters of the model are presented in Table 2. These results demonstrate that the application of the proposed model achieves good agreement between the predictions and observed data, both in growing and inactivating regimes. The improvement in predictive performance is demonstrated by the RMSE values (Table 2) obtained with or without the inclusion of the ζ term, which accounts for the effect of TV-EO. This effect, as indicated by the RMSE values, could be considered negligible at concentrations lower than or equal to the MIC but proves to be significant at higher concentrations. Moreover, the obtained results emphasize the combined effect of TE-EO and temperature on ζ values, indicating that at lower temperatures, the inactivating effect TV-EO is higher, as shown by the results of multiple regression reported in Fig. 1C.

3.4. Antibiofilm activity of TV-EO

The antibiofilm activity was evaluated using a crystal violet staining

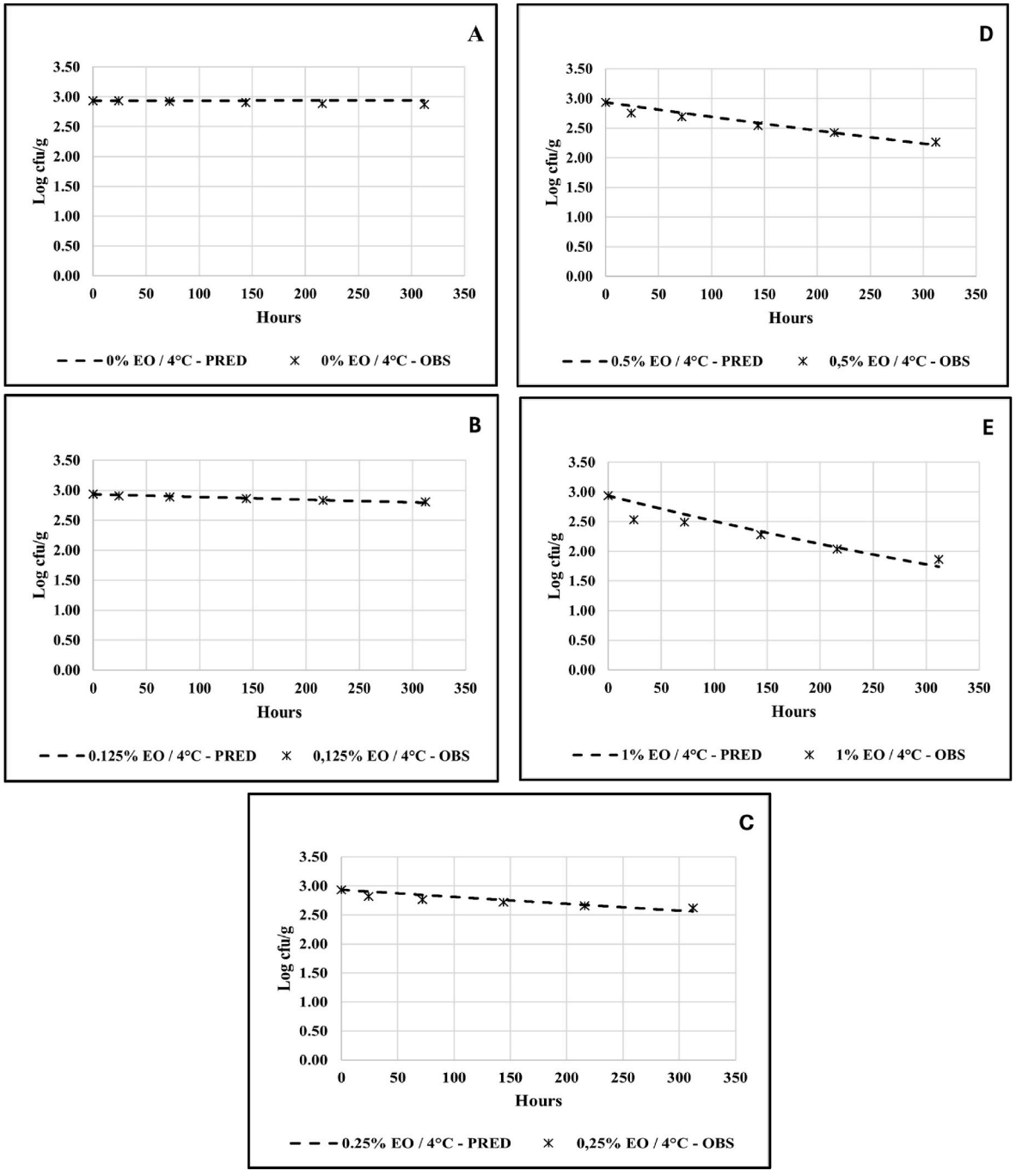

Fig. 3. Observed and predicted behaviour of *L. monocytogenes* for *in situ* tests at 4 °C with 0 % TV-EO (panel A), 0.125 % TV-EO (panel B), 0.25 % TV-EO (panel C), 0.5 % % TV-EO (panel D) and 1 % % TV-EO (panel E).

Table 2
Primary parameters for each predicted curve.

	In vitro tests						*In situ* tests				
% TV EO	0	0.06	0.1	0	0.06	0.1	0	0.125	0.25	0.5	1
T (°C)	10	10	10	20	20	20	4	4	4	4	4
Q_o[a]	0.494	0.494	0.494	0.006	0.006	0.006	0.010	–	–	–	–
N_{max}	9.12	9.12	9.12	8.94	8.94	8.94	8.00	8.00	8.00	8.00	8.00
μ_{max}	0.051	0.051	0.051	0.295	0.295	0.295	0.001	0.001	0.001	0.001	0.001
Z	1.000	0.906	0.766	1.000	0.980	0.817	1.000	−0.258	−0.699	−1.409	−2.502
RMSE without ζ	0.381	0.478	0.743	0.2099	0.228	0.6300	0.039	0.080	0.515	0.707	0.978
RMSE with ζ	0.381	0.414	0.335	0.209	0.224	0.372	0.039	0.011	0.056	0.066	0.145

[a] Q terms was not considered in predictions of decreasing kinetics for mathematical reasons.

assay to assess the ability of TV-EO to inhibit biofilm formation and eradicate existing biofilms (Fig. 1D). Our results demonstrated that TV-EO at MIC and MIC/2 concentrations inhibited biofilm formation by 100 ± 0.00 % and 66.01 ± 1.47 %, respectively, after 24 h of incubation. Furthermore, the eradication of pre-formed biofilms revealed that TV-EO at MIC and MIC/2 concentrations achieved eradication percentages of 91.33 ± 1.23 % and 25.60 ± 3.56 %, respectively, after 24 h of incubation.

3.5. *In silico assessment*

In this study, the antilisterial and anti-biofilm formation activity of TV-EO was assessed by testing its major components against β-ketoacyl-ACP synthase II (fatty acid biosynthesis) and chorismate synthase (biofilm pathway). The results were summarized in Table 3 and Fig. 4.

By targeting β-ketoacyl-ACP synthase II, results revealed that most tested components exhibited lower affinities than the standard inhibitor, platensimycin (−10.2 kcal/mol) (Table 3). However, carvacrol showed a higher affinity than the other molecules, approaching that of platensimycin. The interaction between carvacrol and β-ketoacyl-ACP synthase II reveals several stabilizing bonds influencing the ligand's affinity for the enzyme. A conventional hydrogen bond is observed with ASP42, contributing to the stabilization of the complex and potentially playing a key role in enzyme inhibition. Additionally, hydrophobic interactions involving HIS43, THR207, LEU206, and PRO308 are identified. Notably, a Pi-Pi stacked interaction with HIS43 suggests an additional stabilizing effect through aromatic interactions, while alkyl bonds with LEU206, THR207, and PRO308 further strengthen the anchoring of carvacrol in the active site (Fig. 4). These interactions suggest the inhibitory potential of carvacrol on KAS II, which may disrupt bacterial fatty acid biosynthesis and represent a promising target for the development of new antibacterial agents.

By targeting chorismate synthase, results revealed that most tested components exhibited slightly lower affinity compared to the standard inhibitor, juglone (−4.6 kcal/mol) (Table 3). However, carvacrol displayed an affinity equal to that of juglone, suggesting a notable inhibitory potential. The analysis of interactions between carvacrol and chorismate synthase highlights several stabilizing bonds that are crucial for its affinity toward the enzyme. A Pi-cation interaction with ARG82 plays a central role in stabilizing the complex and may be key to enzymatic inhibition. Additionally, alkyl interactions involving TRP86 and LYS17 further strengthen the anchoring of carvacrol in the active site, reinforcing its potential as a chorismate synthase inhibitor (Fig. 4). These findings suggest that carvacrol could be a promising candidate for the development of new anti-biofilm agents targeting chorismite synthase, a vital protein involved in biofilm pathway.

Table 3
Molecular binding affinities (Kcal/mol) of major components identified in the essential oil of *Thymus vulgaris* with KAS II and Cs.

	Targeted proteins	
	KAS II (PDB: 5SXO)	**Cs** (PDB: 1Q1L)
Compounds	Affinity (Kcal/mol)	
Inhibitor standard	−10.2	−4.6
***p*-Cymene**	−5.8	−4.2
γ-Terpinene	−5.7	−4.5
Thymol	−5.9	−4.3
Carvacrol	−6.9	−4.6

KAS II: β-ketoacyl-ACP Synthase II; **Cs:** Chorismate Synthase.

4. Discussion

The consumption of contaminated food can lead to foodborne illnesses, which pose significant health risks to the population, particularly vulnerable groups such as children, pregnant women, the elderly, and those with weakened immune systems [49]. Therefore, food safety is essential for protecting public health by preventing foodborne illnesses and ensuring that food is safe to consume. During food processing, numerous pathogens can contaminate foods during handling, preparation, processing, and storage [11,50–52]. These pathogens can then be transmitted to consumers, especially if the food is not subjected to effective treatments that inhibit the proliferation of these pathogens [10,53–55]. Today, there is an increasing demand for fast food, particularly uncooked and undercooked foods, which further facilitates the transmission of numerous pathogens to humans.

The contamination of food with *L. monocytogenes* poses a significant public health risk due to its ability to cause a severe infection, listeriosis. EOs have garnered significant attention for their potential use in food safety due to their natural antimicrobial properties [25,34,56]. In this study, we assessed the ability of TV-EO to inhibit *L. monocytogenes* using *in vitro*, *in situ*, and in *silico* approaches. The *in vitro* findings demonstrated that TV-EO exhibits strong activity against *L. monocytogenes*, showing a bactericidal effect as evidenced by the MBC/MIC ration being lower than 4 [32]. In addition, the effect of various TV-EO concentrations on the growth and inactivation kinetics of *L. monocytogenes* was more pronounced at low temperature, suggesting its potential as an antimicrobial food additive for cold storage. Similarly, Mahgoub et al. [57] have demonstrated that the combination of oregano essential oil and cold storage significantly inhibits the growth of *L. monocytogenes* in ready-to-eat smoked turkey meat. Notably, EOs inhibit the growth of pathogenic bacteria through multiple mechanisms, including cell membrane destruction, inhibition of bacterial DNA replication and energy production, and disruption of ribosomal protein synthesis, among others [34].

In the food processing chain, *L. monocytogenes* can resist harsh conditions such as variations of temperature and pH, UV treatment and disinfection, osmotic pressure, and desiccation, allowing it to persist and survive in the food environment, subsequently contaminating food products [58]. To overcome the multiple treatments and environmental stressors, *L. monocytogenes* forms biofilm as an effective mechanism to survive unfavorable conditions [42]. Biofilm formation enables bacteria to adhere to surfaces and produce an extracellular polymeric matrix, creating a protective environment [59]. In the food processing environment, biofilm formation poses a significant challenge to food safety, as bacteria can adhere to different surfaces and form resilient biofilms that resist cleaning and disinfection [60]. In this study, TV-EO

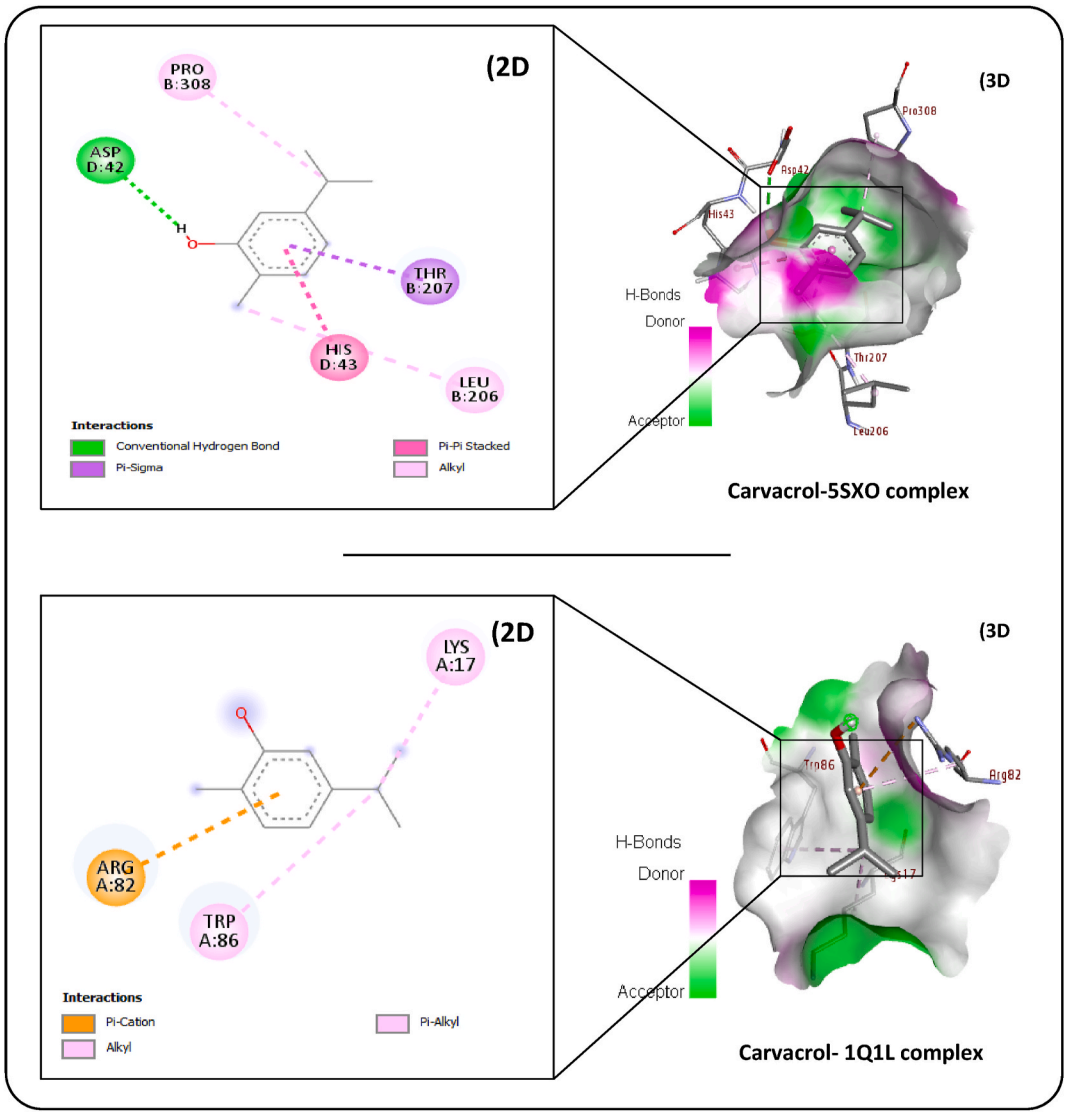

Fig. 4. 2D and 3D Binding Interactions of the compound Carvacrol with Beta-ketoacyl-ACP Synthase II (PDB ID: 5SXO) and Chorismate Synthase (PDB: 1Q1L).

demonstrated a strong capacity to inhibit formation and eradicate pre-formed biofilms, particularly at the MIC value. EOs can inhibit and eradicate biofilm formation through multiple mechanisms by targeting genes involved in different steps of biofilm formation, including bacterial adhesion to surfaces, biofilm formation, and structural integrity of biofilm [61]. The hydrophobic nature of EOs disrupts bacterial cell membranes, leading to leakage of intracellular contents and cell death [60]. Additionally, EOs can interfere with quorum sensing, a key regulatory system for biofilm development, preventing bacterial communication and coordination [60]. Moreover, they can inhibit the production of extracellular polymeric substance, weakening the biofilm structure and making bacteria more susceptible to antimicrobials [60].

Importantly, the antilisterial and antibiofilm effects of TV-EO can act through distinct mechanisms targeting different vital cell sites. Chorismate synthase is an essential enzyme involved in biofilm formation by contributing to the biosynthesis of aromatic amino acids via the shikimate pathway [43,44]. Additionally, β-Ketoacyl-ACP synthase II is a key enzyme involved in fatty acid elongation within the type II fatty acid synthesis system. In microorganisms, this enzyme plays a crucial role in the production of fatty acids that form the membrane phospholipids [45]. Among the tested TV-EO components, our results showed that carvacrol has the highest affinity to these enzymes with comparable binding energy of the reference standards. Carvacrol exhibits strong antibacterial and antibiofilm activities by targeting multiple vital sites within bacterial cells [62]. It disrupts ATP synthesis by inhibiting ATP synthases and interfering with membrane-associated transporters like Na^+/K^+ ATPases [63]. Additionally, carvacrol affects cell wall synthesis by targeting penicillin-binding proteins (PBPs) [62]. It can also influence oxidative stress and energy metabolism by inhibiting key enzymes such as superoxide dismutase (SOD), catalase, and those in the tricarboxylic acid cycle [64]. Furthermore, carvacrol can interfere with quorum sensing and virulence pathways by targeting LuxS protein and affects bacterial stress responses by acting on proteins like Dnak, involved in heat shock regulation [62].

The introduction of TV-EO in poultry minced meat during storage period of 13 days (312 h) demonstrated a significant reduction of *L. monocytogenes* in log cfu/g, with reduction of 1.068 ± 0.132, 0.671 ± 0.091, and 0.317 ± 0.029 for concentrations of TV-EO of 1 %, 0.5 %, and 0.25 %, respectively. These results confirm previous findings reporting the strong ability of TV-EO to mitigate *L. monocytogenes* in food products [65,66]. For instance, de Carvalho et al. demonstrated the potential of TV-EO, added at a concentration of 5 μl/mL to Brazilian coalho cheese, to inhibit *L. monocytogenes* by about 3 log cfu/mL after storage of 24 h at 10 °C [65]. Similarly, Pesavento et al. showed that the addition of TV-EO to meatballs at 0.5 % suppress *L. monocytogenes* concentrations to $<10^2$ CFU/g after a storage period of 14 days at 4 °C [66]. Otherwise, a study

carried out by Solomakos et al. demonstrated that TV-EO had lower inhibitory effects on *L. monocytogenes* in minced beef at 4 °C compared to 10 °C [67], possibly due to matrix interactions or strain variability. In addition to the antimicrobial properties of TV-EO, its richness in flavor compounds, has given it the ability to improve the flavor and odor of food products, which is more appreciated by consumers [36]. Recently, the United States Food and Drug Administration classified TV-EO as generally recognized as safe (GRAS) [68], encouraging its use as natural antimicrobial additive in food products without adverse effects. However, the introduction of TV-EO at high concentrations may alter the original flavor of food products [37,66]. In this regard, Solomakos et al. showed that the introduction of TV-EO at 0.9 % showed unacceptable organoleptic properties in minced meat [67].

Despite these promising results, certain limitations need to be highlighted. The efficacy of TV-EO may vary according to the composition of the food matrix, which may affect its interaction with bacterial cells. Where the presence of proteins, fats and other food components can reduce the bioavailability of TV-EO, thus reducing its efficacy. Furthermore, although *in vitro* and *in situ* experiments suggest strong antibacterial activity, the industrial applications require further validation under real processing conditions. In addition, microbial adaptation and potential resistance mechanisms should be further investigated, as prolonged exposure to sublethal concentrations of TV-EO can induce stress responses in bacteria. Finally, although TV-EO is classified as GRAS, sensory acceptability remains a concern, as higher concentrations may alter the original flavor of food products. Therefore, future studies are needed to optimize TV-EO concentrations in order to balance antimicrobial efficacy and consumer acceptability and assess its long-term efficacy under different storage conditions and packaging systems.

5. Conclusion

This study demonstrates the strong antilisterial potential of TV-EO through comprehensive *in vitro*, *in situ*, and *in silico* approaches. TV-EO exhibited potent bactericidal activity against *L. monocytogenes*, with significant inhibition and eradication of biofilms, highlighting its capacity to target not only planktonic cells but also biofilm-associated bacteria, which are often more resistant to conventional treatments. Predictive modeling further indicated that TV-EO is more effective at lower temperatures, reinforcing its potential for controlling *L. monocytogenes* in refrigerated food products, where the pathogen is commonly found. *In situ* experiments further confirmed the efficacy of TV-EO in reducing bacterial load in minced poultry meat during storage. These findings are particularly relevant for food preservation, where the use of natural antimicrobial agents like TV-EO can provide an additional choice to combat foodborne pathogens. Moreover, molecular docking analysis highlighted the strong binding affinity of key TV-EO components, particularly carvacrol, to essential bacterial enzymes involved in biofilm formation and fatty acid biosynthesis. This suggests that TV-EO works through multiple mechanisms, interfering with crucial cellular processes that support bacterial survival and biofilm development.

Collectively, these findings underscore the potential of TV-EO as a natural antimicrobial agent, offering a promising alternative to conventional preservatives for enhancing food safety and extending shelf life. However, further research is needed to better understand the antilisterial mechanisms of TV-EO. Additionally, the integration of TV-EO into active packaging systems that release EOs in a controlled manner could ensure sustained antimicrobial activity, thereby improving food preservation and extending the shelf life of food products.

CRediT authorship contribution statement

Abdelaziz Ed-Dra: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Luca Nalbone:** Methodology, Investigation, Formal analysis, Data curation. **Abdelaaty A. Shahat:** Writing – review & editing, Validation, Resources, Project administration, Funding acquisition. **Salah Laaraj:** Methodology, Investigation. **Ayoub Farihi:** Visualization, Software, Formal analysis. **Soumia Moujane:** Visualization, Software, Formal analysis. **Omar M. Noman:** Writing – review & editing, Funding acquisition, Data curation. **Kaoutar Elfazazi:** Writing – review & editing, Validation. **Alessandro Giuffrida:** Writing – original draft, Visualization, Software, Data curation, Conceptualization. **Filippo Giarratana:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

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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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Data availability

Data will be made available on request.

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