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Evaluation of natural compounds against *Listeria innocua:* Translating *in vitro* success to processed meat models

Vesna Milanović [a], Mariana Mariz [b], Federica Cardinali [a,*], Cristiana Garofalo [a], Mila Radan [c], Tea Bilušić [c], Lucia Aquilanti [a], Luís Miguel Cunha [b], Andrea Osimani [a]

[a] *Dipartimento di Scienze Agrarie, Alimentari ed Ambientali, Università Politecnica delle Marche, via Brecce Bianche, 60131, Ancona, Italy*
[b] *GreenUPorto/Inov4Agro, Department of Geosciences, Environment and Planning, Faculty of Sciences, University of Porto, Porto, 4169-007, Portugal*
[c] *Department of Biochemistry, University of Split, Ruđera Boškovića 35, Split, 21000, Croatia*

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A B S T R A C T

Ensuring food safety is a significant challenge in the processed meat industry. *Listeria monocytogenes* represents a persistent threat due to its ability to grow in adverse conditions. Nowadays, there's a growing interest in natural preservatives to inhibit its proliferation in foods. Accordingly, the aim of the present study was to preliminarily evaluate the *in vitro* inhibitory activity of various natural antimicrobial agents against *Listeria innocua*, used as a surrogate for *L. monocytogenes*. Thyme essential oil (EO) emerged as the most effective candidate for further *in vivo* testing in pork minced meat with 40% fat and no additional fat, deliberately contaminated with *L. innocua*. The samples were stored for 20 days under two different temperature conditions, mimicking common procedures used to produce salami with short ripening periods and high fat content, such as the Italian *Ciauscolo* PGI salami, either through spontaneous fermentation or with the use of starter cultures. *In vivo*, the inhibitory effect of thyme EO was minimal or absent, regardless of fat content. Statistically significant decreases in *L. innocua* counts (<2%) were sporadic and observed solely in samples stored under temperature conditions mimicking the typical procedure for spontaneously fermented salami production. However, this effect was not sustained until the end of the experiment. While thyme EO demonstrated potential as an antilisterial agent *in vitro*, our findings highlight the complex interaction between antimicrobial agents and food matrices, revealing challenges in practical applications. This underscores the importance of further investigation elucidating the effectiveness of antimicrobial agents in real food systems.

1. Introduction

The genus *Listeria* consists of Gram-positive facultative anaerobic bacteria that are widely distributed in numerous environments, such as soil, water, and various food sources. The most prevalent *sensu stricto* species of this genus found in foods are *Listeria monocytogenes* and *Listeria innocua* (Collins et al., 1991; Raschle et al., 2021). *L. monocytogenes* is of particular concern as it can cause listeriosis, a severe foodborne illness that primarily affects vulnerable populations, including pregnant women, newborns, the elderly, and individuals with weakened immune systems (Donovan, 2015). *L. monocytogenes* poses a significant threat in the food industry due to its remarkable survival abilities across a wide range of environmental conditions, including temperature, pH, and salt concentration (Matle et al., 2020; Osimani & Clementi, 2016; Wiktorczyk-Kapischke et al., 2021). This resilience enables it to persist in food production chains and refrigerated products for extended periods (Lakicevic et al., 2022; Matle et al., 2020). Due to the pathogenicity and persistent nature of *L. monocytogenes*, researchers often turn to *L. innocua*, a non-pathogenic *Listeria* species found in similar environments, as a surrogate for research purposes (Mohan et al., 2019). *L. innocua* does not cause illness in humans but shares similar genotypic and phenotypic characteristics with *L. monocytogenes*, making it suitable for mimicking its behaviour and survival patterns in food processing environments (Mohan et al., 2019). This substitution allows researchers to conduct controlled studies safely and ethically, maintaining research integrity and minimizing the risk of contamination (Mohan et al., 2019). Furthermore, the use of *L. innocua* as a surrogate has been widely accepted in scientific literature for assessing the antimicrobial properties of food additives and preservatives (Bonilauri et al., 2021; Cenci-Goga et al., 2018; Evrendilek & Balasubramaniam, 2011; Hospital et al., 2012;

* Corresponding author.
E-mail address: f.cardinali@univpm.it (F. Cardinali).

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Lopez et al., 2021; Lorentzen et al., 2010).

Numerous studies have reported a correlation between different types of meat and human listeriosis outbreaks on a global scale (Center for Disease Control and Prevention, 2020; EFSA Panel on Biological Hazards, 2019; Matle et al., 2020, EFSA Panel on Biological Hazards, 2019; Osimani and Clementi, 2016). Minced meat, a common ingredient in many meat-based dishes and processed products, provides an ideal environment for the growth of *L. monocytogenes,* mainly due to increased surface area during grinding and potential contamination during handling (Kiprotich & Aldrich, 2022). Fermented salami is traditionally prepared using minced meat as the primary ingredient. The process begins with the selection of meat, which is then subjected to chopping, mincing, and mixing with curing ingredients, spices, and additives (Branciari et al., 2020). The safety of the final product is ensured by specific factors and physicochemical conditions, such as the presence of sodium chloride, nitrates, and nitrites, lower pH values, and decreased water activity (a_w) (Branciari et al., 2020). Lactic acid bacteria (LAB), Gram-positive Catalase-positive Cocci (GCC+), and to a lesser extent, yeasts and moulds play a fundamental role in the production of fermented sausages (Aquilanti et al., 2007; Delgado et al., 2023; Rouhi et al., 2023).These microorganisms, which can be naturally present in the meat or introduced through the inoculation of starter cultures, contribute significantly to safety, flavour development, and the overall quality of the final product through fermentation and acidification processes (Aquilanti et al., 2007; Delgado et al., 2023). However, it's worth noting that specific physicochemical conditions in fermented salami may create a favourable environment for *Listeria* growth, especially in products with short ripening periods and high fat content such as the Italian *Ciauscolo* Protected Geographical Indication (PGI) salami (Regulation (EC)) (Belleggia et al., 2020; Branciari et al., 2016; Osimani et al., 2023; Petruzzelli et al., 2010; Ranucci et al., 2013).

In recent years, due to concerns about bacterial antibiotic resistance, a lack of new drug development, and the use of synthetic additives, there has been a growing interest in finding effective alternatives, in particular, natural preservatives, against foodborne pathogens (Behbahani et al., 2021; Chouhan et al., 2017). Essential oils (EOs) are a promising alternative with potent antimicrobial, antioxidant, and flavour-enhancing properties (Ali et al., 2022). Derived from various parts of plants, these compounds contain a wide range of bioactive substances that have shown effectiveness against foodborne pathogens, including both *L. monocytogenes* and its surrogate, *L. innocua* (Ali et al., 2022; Behbahani et al., 2013; Behbahani et al., 2021; Castro et al., 2018; Chouhan et al., 2017; Evrendilek & Balasubramaniam, 2011; Jalil Sarghaleh et al., 2023; Sureshjani et al., 2014; Tabatabaei Yazdi & Alizadeh Behbahani, 2013; Yeganegi et al., 2018). Integrating EOs into processed meat formulations could represent a natural and safe method to enhance microbial safety and prolong their shelf life (Alghooneh et al., 2015; Heydari et al., 2020; Noshad et al., 2021; Tanavar et al., 2021). However, it's important to note that the efficiency of EOs in inhibiting bacterial growth in food model systems can be influenced by several factors, including the capacity of bacteria to form biofilms, interactions with suspended organic substances, proteins, and fat, availability of nutrients, and water content, all potentially diminishing the effectiveness of EOs compared to *in vitro* studies (Gurtler & Garner, 2022; Vidaković Knežević et al., 2023).

In the European Union food market, ensuring the safety of foodstuffs is pivotal [Regulation (EC)]. Commission Regulation (EC) No 2073/2005, amended by Commission Regulation (EC) 1441/2007, establishes microbiological criteria for foodstuffs. It mandates testing the growth and survival of *L. monocytogenes* in ready-to-eat foods under different processing/storage conditions. Moreover, processing areas and equipment should be sampled and analysed for *L. monocytogenes* to verify its absence from surfaces.

Further research is needed to comprehensively investigate the inhibitory activity of EOs against *L. monocytogenes* in processed meat model systems, as well as their specific impacts on the fermentation process and sensory characteristics of fermented meats.

Accordingly, the aim of the present study was to preliminarily evaluate *in vitro* the inhibitory activity of different natural antimicrobial agents against *L. innocua*, used as a surrogate for *L. monocytogenes*. Subsequently, the practical application of the most potent antimicrobial agent was assessed *in vivo*, utilizing pork minced meat samples, both with and without added fat, deliberately contaminated with *L. innocua*. The samples were maintained for 20 days under two different temperature conditions, thus mimicking the procedures used to produce Italian PGI *Ciauscolo* salami; either fermented spontaneously or using starter cultures. This specific type of salami was selected as a model due to its short ripening period and high fat content, factors that heighten the risk of *Listeria* contamination. Additionally, we examined whether the selected antimicrobial agents had any inhibitory effect on the growth of beneficial microorganisms (LAB, GCC+, yeasts) typically involved in the production of fermented salami. This research provides valuable insights for enhancing the safety and quality of processed meat products by addressing food safety, microbial balance, and exploring alternative preservatives.

2. Materials and methods

2.1. Microbial strains and growth conditions

L. innocua strains (LI1, LI2, and LI3) were previously isolated from raw meat samples; LI1 and LI2 were kindly provided by the Department of Agricultural, Forest and Food Sciences (DISAFA) from the University of Turin (Italy), whereas the strain LI3 was kindly provided by Institute for Experimental Veterinary Medicine of Umbria and Marche, Perugia (Italy). LAB including *Latilactobacillus sakei* (P8-1, P13-2, SP1-3, SP6-4, SP7-3) and *Latilactobacillus curvatus* (P2-4, P8-4, P14-1, SP2-3, SP7-4) cultures were obtained from the *Painho* sausages (P) (Belleggia, Ferrocino, Reale, et al., 2022) and fermented fish sausages (SP) (Belleggia, Ferrocino, Rita Corvaglia, et al., 2022). *Staphylococcus xylosus* was previously isolated from commercial meat starter culture, whereas *Debaryomyces hansenii* cultures (56, 64, 65, 85, 92, 94) were isolated from buffalo milk yoghurt (Milanović et al., 2021). All the strains were maintained at the Culture Collection of the Department of Agriculture, Food and Environmental Science (Università Politecnica delle Marche, Ancona, Italy) at −80 °C as glycerol stocks and revitalised by culturing them in appropriate growth media; De Man Rogosa and Sharpe (MRS) (VWR International Srl, Milan, Italy) for 48 h at 30 °C for the LAB, Brain Heart Infusion (BHI) (VWR International Srl.) for 24 h at 37 °C for *L. innocua* and *S. xylosus*, and Yeast extract Peptone Dextrose (YPD) for 48 h at 25 °C for *D. hansenii* strains. All strains were previously identified at the molecular level by 16S rRNA (bacteria) or ITS1-5.8S-ITS2 rRNA (yeasts) gene sequencing of extracted DNA.

2.2. Antimicrobial agents

Cinnamon (*Cinnamonum zeylanicum*), clove (*Syzygium aromaticum*), cumin seed (*Cuminum cyminum*), garlic (*Allium sativum*), ginger (*Zingiber officinale*), lemon (*Citrus limon*), lemongrass (*Cymbopogon citratus*), lime (*Citrus aurantifolia*), mandarin (*Citrus reticulata*), orange (*Citrus sinensis*), rosemary (*Salvia rosmarinus*), and thyme (*Thymus vulgaris*) EOs were obtained from Sigma Aldrich (Saint Louis, Missouri, USA). Basil EO (*Ocimum basilicum*) was obtained from Biover NV Sucursal en España (León, Spain), cardamom EO (*Elettaria cardamomum*) from Ladrôme Laboratoire (Saillans, France), black pepper EO (*Piper nigrum*) from Erboristeria Magntina S.r.l. (Turin, Italy), and sage EO (*Salvia officinalis*) from Equilalud S.L.U. (Huerte-pamplona, Spain). The quality parameters of EOs were described in an accompanying technical report. Oleacein (17 ppm), oleocanthal (14 ppm), and nootkatone 300 were kindly provided by the Department of Biochemistry from the University of Split (Croatia).

2.3. Disk diffusion assay

A disc diffusion assay was used to determine the antimicrobial activity of the above-listed EOs and organic compounds against *L. innocua*, *Lat. sakei*, *Lat. curvatus*, *S. xylosus*, and *D. hansenii* strains. The strains were subcultured two times in 10 mL of appropriate growth media, and the cells from each culture were collected by centrifugation at 4186×*g* for 5 min at room temperature using a Rotofix 32A centrifuge (Hettich, Tuttlingen, Germany). The cell pellets were resuspended in a sterile physiological solution (0.85% NaCl). The concentration of cells in each suspension was determined using a Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) by measuring the optical density (OD) at 600 nm, and further diluted with a sterile physiological solution to reach a final concentration of 6 and 8 Log cells/mL for yeasts and bacteria, respectively. For each tested microorganism, 100-μL aliquots were distributed on Petri plates containing appropriate solid growth media. Nineteen sterile filter paper discs (0.6 cm diameter) (Schleicher & Schuell BioScience GmbH, Dassel, Germany) were distributed uniformly among four agar plates for each strain and soaked with 10 μL of the tested antimicrobial component. The plates were incubated under the same conditions as described above for each microorganism. The disc diffusion assay was performed in duplicate, and the results were expressed as the average value (cm) ± standard deviation. The best performing antimicrobial agent was selected for further analyses.

2.4. Minimum inhibitory concentration (MIC) determination

Thyme EO was identified as the best performing antimicrobial agent after the disk diffusion assay. Therefore, its minimum inhibitory concentration (MIC) was determined against strains of *L. innocua*, as well as selected strains of *Lat. curvatus*, *Lat. sakei*, *S. xylosus*, and *D. hansenii* using the broth microdilution method. The 100-μL aliquots of appropriate growth media for each tested microorganism were distributed in sterile 96-well U-pure grade S-clear microdilution plates (Brand Gmbh, Wertheim, Germany). For the susceptibility testing, thyme EO was twofold serially diluted in the same growth medium to obtain concentrations ranging from 250 μL/mL to 0.24 μL/mL. The strains were preliminary grown under the conditions described above. The concentration of each culture was determined spectrophotometrically and further diluted to reach a concentration of about 5 Log cells/mL for bacteria and 6 Log cells/mL for yeasts. Hence, 100-μL aliquots were distributed in microdilution plates containing thyme EO in decreasing concentrations. A positive control (inoculated growth media without EO) was included for each tested strain to validate adequate microbial growth during the incubation period. The microplates were aseptically closed and incubated as described above for each microorganism. The MIC was defined as the lowest concentration of the thyme EO able to inhibit visible microbial growth at the end of the incubation period. All tests were performed in duplicate.

2.5. Experimental design and preparation of the samples

Four kilograms of pork meat without visible fat were purchased directly from the butcher's shop and transported immediately to the laboratory in refrigerated conditions. After arrival, the meat surface was sterilized by exposure to a gas torch burner (Kemper group, Parma, Italy) and minced in sterile conditions using a sterilized meat grinder Duetto Plus (R.G.V. s.r.l., Cermenate, Italy). To prevent microbial contamination of the samples, all procedures were conducted within the vicinity of two Bunsen burners. Sterile spoons, bowls, and gloves were utilized for sample handling, and rigorous cleaning and disinfection protocols were implemented for the work surfaces. The minced meat was added with salt (2.7% w/w), mixed well, and then divided into two equal portions, each weighing 2 kg. One portion was added with 40% fat to reproduce the model of salami characterized by short ripening periods and high fat content (e.g., the Italian PGI *Ciauscolo* salami) (Belleggia et al., 2020; Osimani et al., 2023), while the other portion remained without any additional fat. To evaluate the inhibitory activity of thyme EO against *L. innocua*, both sample types were further divided into two groups (1 kg each). Each group consisted of three 150 g aliquots subjected to different treatments: i) untreated (control), ii) inoculated with *L. innocua*, and iii) inoculated with *L. innocua* and added with thyme EO. Controls were included to monitor for any signs of cross-contamination and to ensure the validity of the experimental results. The samples were placed in sterile Petri dishes and stored for 20 days at different temperatures according to the recommendations for the production of *Ciauscolo* PGI salami (Belleggia et al., 2020; Osimani et al., 2023). Those from the first group were kept at 4 °C for 6 days and then at 10 °C until the end of trial, thus simulating typical procedure for the production of spontaneously fermented salami, whereas the samples from the second group were initially stored at 20 °C followed by a daily temperature decrease (1 °C) until reaching 14 °C, upheld until the end of trial, thus simulating typical procedure for the production of salami fermented by the addition of starter cultures (Fig. 1). The experiment was performed in duplicate. The samples were collected for further analysis immediately after preparation (t0) and after 3 (t3), 6 (t6), 10 (t10) and 20 (t20) days of storage.

2.6. Listeria innocua inoculation and thyme EO addition

According to French Agency for Food, Environmental and Occupational Health & Safety (ANSES, 2019) recommendation to inoculate not less than two different strains when microbial food challenge tests are performed, three strains of *L. innocua* were used in the present study. The strains were grown separately for 24 h at 37 °C in tubes containing 10 mL of BHI broth (Sigma, Milan, Italy) and then centrifuged at 4186×*g* for 5 min at room temperature to precipitate the cells. The supernatant was discarded, and the cells were resuspended in a sterile physiological solution. The concentration of the cells in each suspension was determined spectrophotometrically, whereas cell viability was assessed by viable counts on BHI agar. Finally, equal parts of the three *L. innocua* strains were mixed to reach a final concentration of 8 Log cells/mL. The inoculum was set at 1% of each substrate (150 g) as recommended by ANSES (2019). Accordingly, 1.5 mL aliquots of the final bacterial suspension (8 Log cells/mL) were inoculated in minced meat samples to reach a final load of about 6 Log cells/g, and homogeneously distributed by mixing for 5 min with a sterile tablespoon.

To test the efficiency of thyme EO against *L. innocua*, the minced meat substrates (150 g) inoculated as described above were supplemented with 1.95 μL/g thyme oil, in accordance with MIC assay results, and mixed well for 5 min using a sterile tablespoon to uniformly distribute the EO.

2.7. pH and water activity measurements

The pH of the samples during storage was measured using a pH meter equipped with a HI2031 solid electrode (Hanna Instruments, Padova, Italy). The a_w was measured using an AwTherm apparatus (Rotronic, Bassersdorf, Switzerland) in accordance with the ISO 21807:2004 standard method. The results of two biological and two technical replicates were expressed as the mean value ± standard deviation.

2.8. Viable counts

To perform the viable counts of *L. innocua* in minced meat samples prepared as described above, 5 g aliquots were collected during storage (t0, t3, t6, t10, t20) at different temperatures, added with 45 mL sterile physiological solution, and homogenized with a Stomacher apparatus (VWR International Srl.) at 260 rpm for 5 min. The obtained homogenates (1/10 dilution) were further ten-fold serially diluted and inoculated using the spread plate technique on Agar Listeria according to

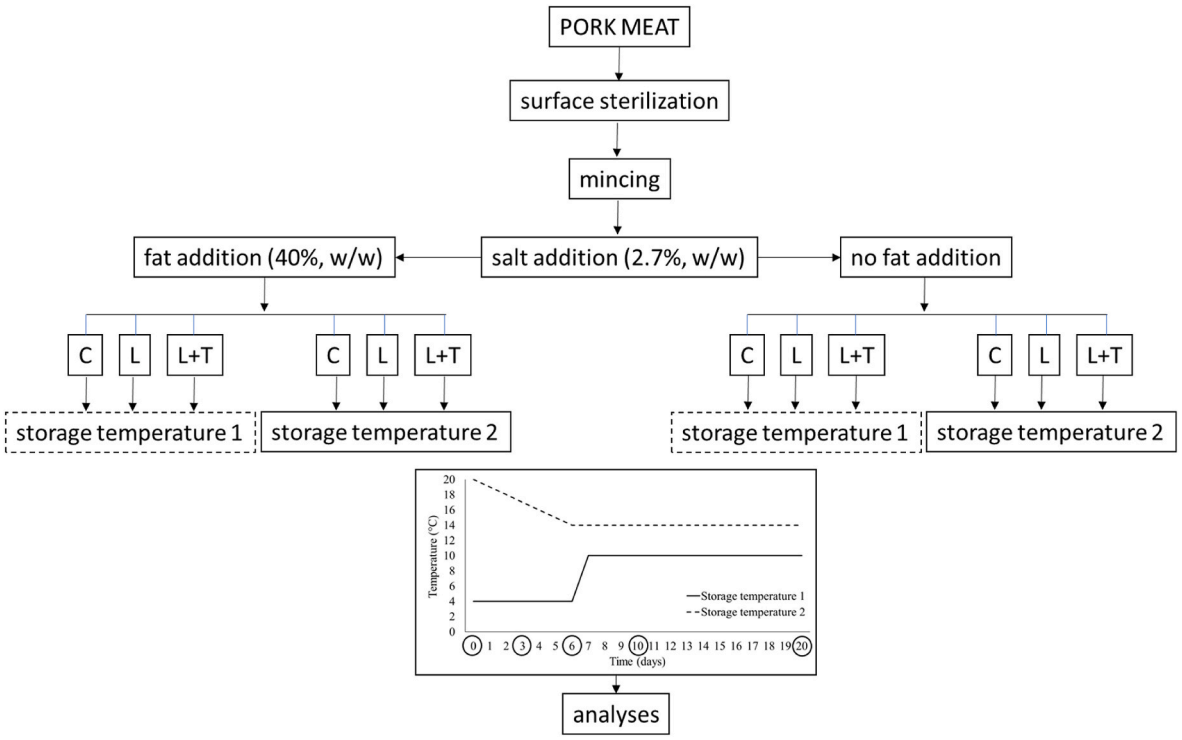

Fig. 1. Experimental design C, control (untreated) samples; L, samples inoculated with *Listeria innocua*; L + T, samples inoculated with *L.innocua* and treated with thyme essential oil.

Ottaviani & Agosti (ALOA) (VWR International Srl.). The plates were incubated at 37 °C for 24–48 h and the results of two biological and two technical replicates were expressed as Log cfu/g and reported as the mean value ± standard deviation.

2.9. Statistical analysis

The Tukey Honest Significant Difference (HSD) test, with a significance level of $P < 0.05$, was employed to assess variations among samples through one-way analysis of variance (ANOVA) utilizing JMP® Version 11.0.0 software (SAS Institute Inc., Cary, NC).

3. Results and discussion

3.1. Disk diffusion assay

The disc diffusion assay was conducted prior to assess the antimicrobial activities of the sixteen EOs (basil, black pepper, cardamom, cinnamon, clove, cumin seed, garlic, ginger, lemon, lemongrass, lime, mandarin, orange, rosemary, sage, and thyme) and three tested organic compounds (oleacein, oleocanthal, and nootkatone) against *L. innocua*, as well as against LAB, GCC+, and yeast cultures of the species that may be naturally present in meat or introduced through the inoculation of starter cultures. These microorganisms significantly contribute to safety, flavour development, and the overall quality of the final product through fermentation and acidification processes (Aquilanti et al., 2007; Delgado et al., 2023; Rouhi et al., 2023). Therefore, they underwent screening to prevent the selection of antimicrobial components that could negatively influence the growth of these beneficial microorganisms. The average results, expressed in centimetres of growth inhibition zone for each species represented by different strains, are reported in Table 1. Additionally, the results for each strain separately are presented in Table 2. It is noteworthy that the disk diffusion method is frequently used as a preliminary evaluation of antibacterial activity before conducting more extensive studies (Balouiri et al., 2016). Variables such as the volume of EO applied to paper disks, the thickness of the agar layer, and the use of solvents show significant diversity across various studies, rendering the comparison of published data quite unfeasible (Burt, 2004). In the present study, the statistical analysis revealed that thyme EO exhibited the highest inhibitory activity (3.97 ± 0.31 cm) against *L. innocua* strains, followed by cinnamon (2.88 ± 0.32 cm) and lemongrass EOs (1.70 ± 0.15 cm). Thyme EO was also the most efficient in inhibiting the growth of the tested beneficial bacteria (*Lat. sakei*, *Lat. curvatus*, and *S. xylosus*). This is in line with previous findings showing the good ability of thyme EO to inhibit the growth of both Gram-positive and Gram-negative bacteria (Kowalczyk et al., 2020). The garlic EO had the strongest inhibitory activity of *D. hansenii* strains. Garlic has been extensively studied for its antimicrobial properties, primarily due to allicin, a main compound in crushed garlic that has demonstrated broad antimicrobial activities against different yeast species, including *D. hansenii* (Fufa, 2019; Kim et al., 2021). Among the tested organic compounds, nootkatone showed the highest inhibitory activity against all tested microorganisms. Nootkatone, a sesquiterpene found in citrus peels, particularly in grapefruits, is synthesized through the oxidation of its precursor, valencene (Leonhardt & Berger, 2015). Yamaguchi (2019) reported the antibacterial activity of this compound against some Gram-positive bacteria, including those from *Staphylococcus* and *Listeria* genera.

3.2. Minimum inhibitory concentration of thyme EO

Thyme EO demonstrated the ability to inhibit the growth of *L. innocua* strains at concentrations lower (0.98–1.95 μL/mL) than those required to inhibit the growth of most tested beneficial microorganisms (Table 3). These latter microorganisms were inhibited by thyme EO at a concentration of 3.91 μL/mL, except for the *S. xylosus* strain, which was inhibited at a concentration of 0.98 μL/mL. The slight discrepancy in results between the disk diffusion assay and MIC can be attributed to several factors, including the variations in microbial growth in liquid versus solid media, the extent of microbial exposure to EO, EO solubility,

Table 1
The activity of natural antimicrobial agents against *Listeria innocua, Latilactobacillus sakei, Latilactobacillus curvatus, Staphylococcus xylosus,* and *Debaryomyces hansenii* strains, as detected by disk diffusion assay.

Antimicrobial agents	Growth inhibition zone (cm)*				
	L. innocua	*D. hansenii*	*Lat. sakei*	*Lat. curvatus*	*S. xylosus*
Lime EO	0.40 ± 0.16^{efg}	0.98 ± 0.05^{ef}	1.22 ± 0.34^{cd}	1.31 ± 0.33^{c}	0.65 ± 0.07^{fg}
Ginger EO	0.17 ± 0.29^{fg}	0.06 ± 0.14^{f}	1.07 ± 0.28^{cd}	1.08 ± 0.19^{c}	0.35 ± 0.49^{gh}
Lemongrass EO	1.70 ± 0.15^{c}	3.38 ± 1.42^{d}	1.42 ± 0.39^{bc}	1.24 ± 0.41^{c}	2.45 ± 0.07^{c}
Mandarin EO	0.52 ± 0.53^{efg}	0.39 ± 0.37^{ef}	0.00 ± 0.00^{d}	0.09 ± 0.20^{e}	0.00 ± 0.00^{h}
Cinnamon bark EO	2.88 ± 0.32^{b}	4.69 ± 0.46^{bc}	2.48 ± 0.84^{b}	2.76 ± 0.72^{b}	3.40 ± 0.42^{b}
Orange EO	0.25 ± 0.23^{efg}	0.97 ± 0.32^{ef}	1.15 ± 0.28^{cd}	1.05 ± 0.39^{c}	0.00 ± 0.00^{h}
Lemon EO	0.17 ± 0.29^{fg}	0.78 ± 0.28^{ef}	0.69 ± 0.63^{cd}	0.21 ± 0.29^{de}	0.75 ± 0.07^{fg}
Clove EO	1.42 ± 0.16^{cd}	2.88 ± 0.20^{d}	1.50 ± 0.45^{bc}	1.44 ± 0.24^{c}	1.30 ± 0.14^{de}
Cumin seed EO	0.87 ± 0.13^{defg}	4.03 ± 1.51^{cd}	1.35 ± 0.27^{bc}	1.15 ± 0.23^{c}	1.45 ± 0.07^{d}
Garlic EO	0.95 ± 0.05^{cde}	8.50 ± 0.00^{a}	1.56 ± 1.41^{bc}	0.71 ± 0.44^{cde}	1.65 ± 0.64^{d}
Thyme EO	3.97 ± 0.31^{a}	5.29 ± 0.62^{b}	3.87 ± 1.13^{a}	3.62 ± 0.75^{a}	4.80 ± 0.14^{a}
Rosemary EO	0.58 ± 0.32^{efg}	0.92 ± 0.06^{ef}	1.19 ± 0.36^{cd}	1.06 ± 0.19^{c}	0.90 ± 0.00^{ef}
Sage EO	0.92 ± 0.23^{def}	1.06 ± 0.18^{ef}	1.34 ± 0.33^{bc}	1.27 ± 0.22^{c}	0.90 ± 0.00^{ef}
Cardamom EO	0.82 ± 0.06^{defg}	0.84 ± 0.14^{ef}	1.42 ± 0.24^{bc}	1.35 ± 0.18^{c}	0.90 ± 0.00^{ef}
Basil EO	0.62 ± 0.23^{efg}	1.41 ± 0.63^{e}	1.00 ± 0.20^{cd}	0.98 ± 0.17^{cd}	0.85 ± 0.07^{efg}
Black pepper EO	0.15 ± 0.26^{fg}	0.06 ± 0.14^{f}	1.33 ± 0.22^{bc}	1.19 ± 0.22^{c}	0.00 ± 0.00^{h}
Oleacein	0.13 ± 0.23^{g}	0.13 ± 0.19^{f}	0.78 ± 0.07^{cd}	0.83 ± 0.27^{cde}	0.75 ± 0.07^{fg}
Oleokantal	0.13 ± 0.23^{g}	0.22 ± 0.17^{f}	0.80 ± 0.19^{cd}	0.71 ± 0.28^{cde}	0.75 ± 0.07^{fg}
Nootkatone	0.80 ± 0.13^{defg}	0.08 ± 0.18^{f}	1.78 ± 0.23^{bc}	0.86 ± 0.21^{cde}	0.75 ± 0.07^{fg}

EO, essential oil; *Diameters (cm) of growth inhibition zones expressed as the mean value of the isolates from the same species ± standard deviation. Means followed by different letters within each column (a, b, c …) indicate significant differences ($P < 0.05$) between essential oils for each species.

and their diverse origins (Ballester-Costa et al., 2013). Furthermore, the comparison of MIC values across various studies may be difficult due to the employment of non-standardized quantitative or semiquantitative methods, variations in microbial strains utilized, differences in the solubility of various EO compounds in liquid mediums, and disparities in EO compositions (Djenane et al., 2011; Thielmann et al., 2019). However, the MIC values obtained for thyme EO against *L. innocua* strains fall within the range of results reported by other authors for *L. monocytogenes* strains. Some studies reported higher MIC values (2.5–5 μL/mL) (Gouveia et al., 2016; Mazzarrino et al., 2015; Sokovic et al., 2010), whereas others reported lower MIC values (0.12–1 μL/mL) (Carvalho et al., 2015; Cosentino et al., 1999; Firouzi et al., 1998; Vidaković Knežević et al., 2023).

3.3. Efficacy of thyme EO against Listeria innocua in vivo

Among the tested natural antimicrobial agents, thyme EO exhibited the highest inhibitory activity against *L. innocua* strains *in vitro*. Moreover, thyme EO demonstrated the ability to inhibit the growth of *L. innocua* strains at concentrations lower than those required to inhibit the growth of most tested beneficial microorganisms, such as LAB, GCC+, and yeasts, which are typically involved in the production of fermented salami (Aquilanti et al., 2007; Delgado et al., 2023). Furthermore, due to thyme's common usage as a highly valued flavouring agent with a spicy taste, often incorporated into various types of meats (Nieto, 2020), it was chosen to evaluate its efficacy as an antimicrobial agent *in vivo*. This evaluation was conducted using pork minced meat with or without added fat, maintained under two different temperature conditions (Fig. 1), thus mimicking the procedures employed in producing Italian PGI *Ciauscolo* salami, either through spontaneous fermentation or using starter cultures (Belleggia et al., 2020; Osimani et al., 2023). This particular type of salami was chosen as a model due to its short ripening period and high fat content, both factors that increase the risk of *Listeria* contamination. To evaluate the inhibitory effects of thyme EO on *L. innocua in vivo*, the minced meat samples with or without added fat were divided into three aliquots, each undergoing distinct treatments: i) untreated (control), ii) inoculated with *L. innocua*, and iii) inoculated with *L. innocua* and supplemented with 1.95 μL/g thyme EO, in accordance with MIC assay results (Fig. 1).

The results of the viable counts of *L. innocua* assessed at different time points: immediately after inoculation (t0), and subsequently at 3 (t3), 6 (t6), 10 (t10), and 20 (t20) days during storage are presented in Table 4. Overall, the counts of *L. innocua* in all samples, regardless of fat addition, stored at temperature 1 (Fig. 1) to simulate the typical production of spontaneously fermented salami showed a slight decrease during the initial 10 days of storage, followed by a notable increase by the end of the experiment (t20). In contrast, samples stored at temperature 2 (Fig. 1), simulating the typical salami production procedure using starter cultures, consistently showed an increase in *L. innocua* load from t0 to t20. This was expected, given that these storage conditions involve relatively high temperatures ranging from 14 to 20 °C which are closer to the ideal conditions for *Listeria* growth, typically between 30 and 37 °C (Batt, 2014).

Concerning the inhibitory activity of thyme EO against the proliferation of *L. innocua* in the samples kept at storage temperature 1 (Fig. 1), inhibitory effects were observed only at a few sampling time points. In the samples without added fat, a modest but statistically significant decrease in viable *L. innocua* cells (<2%) was observed only after 10 days of storage (t10), whereas a similar effect in the samples containing 40% fat was exclusively observed at the t6 sampling point. However, in both cases, the slight inhibitory effect was not maintained until the end of the storage period (t20).

Concerning the samples stored at storage temperature 2 (Fig. 1), no inhibitory activity of thyme EO was observed. Unpredictably, the samples supplemented with thyme EO exhibited a higher load of *L. innocua* compared to the respective samples contaminated with *L. innocua* at t3 and t20 sampling points for samples without added fat, as well as t10 point for samples containing 40% fat.

Our findings are consistent with a limited number of studies indicating either minimal or no antimicrobial activity of thyme EO against *Listeria* or other pathogens *in vivo*. For instance, Gouveia et al. (2016) investigated the antimicrobial properties of thyme EO against *L. monocytogenes* in beef samples stored at 2 and 8 °C over a 28-day storage period. The results of Gouveia et al. (2016) revealed that this EO, at a concentration of 3.9 μL/mL, did not cause a significant reduction in the *L. monocytogenes* load compared to samples without thyme EO addition. Likewise, Solomakos et al. (2008) reported that the addition of 0.6% (v/w) thyme EO did not exhibit inhibitory effects against *Escherichia coli* strains in minced beef meat stored at 4 °C. Potential reasons for thyme EO's failure to inhibit *Listeria* growth in minced meat despite promising plate assay results include its instability, volatility, low solubility in water, decreased effectiveness when interacting with the meat matrix, pH and temperature effects, strain resistance, concentration issues, and synergistic or antagonistic interactions with other compounds (Posgay et al., 2022).

Regarding interactions with various food components, Burt (2004) reported that the interaction between the hydrophobic constituents of EOs and fat may reduce their antimicrobial effectiveness. However, in

Table 2
The activity of natural antimicrobial agents against *Listeria innocua, Latilactobacillus sakei, Latilactobacillus curvatus, Staphylococcus xylosus,* and *Debaryomyces hansenii* strains, as detected by disk diffusion assay.

Antimicrobial agents	Growth inhibition zone (cm)*																		
	D. hansenii						*Lat. sakei*					*Lat. curvatus*					*S. xylosus*	*L. innocua*	
	56	64	65	85	92	94	P8-1	P13-2	SP1-3	SP6-4	SP7-3	P2-4	P8-4	P14-1	SP2-3	SP7-4		LI1	LI2	LI3
Lime EO	1.00 ± 0.42	0.90 ± 0.14	1.00 ± 0.28	1.05 ± 0.49	0.95 ± 0.07	0.95 ± 0.07	1.40 ± 0.85	1.70 ± 0.85	1.20 ± 0.28	0.85 ± 0.21	0.95 ± 0.35	0.90 ± 0.00	1.50 ± 0.71	1.25 ± 0.35	1.75 ± 0.21	1.15 ± 0.78	0.65 ± 0.07	0.90 ± 0.42	0.00 ± 0.00	0.30 ± 0.42
Ginger EO	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.35 ± 0.49	0.00 ± 000	0.00 ± 0.00	0.95 ± 0.35	1.05 ± 0.21	0.85 ± 0.07	0.95 ± 0.35	1.55 ± 0.64	1.20 ± 0.28	1.30 ± 0.49	1.10 ± 0.14	1.00 ± 0.14	0.80 ± 0.28	0.35 ± 0.49	0.50 ± 0.71	0.00 ± 0.00	0.00 ± 0.00
Lemongrass EO	3.40 ± 1.56	3.30 ± 0.99	3.80 ± 2.40	5.50 ± 0.99	3.20 ± 2.12	1.05 ± 1.48	1.30 ± 0.14	1.05 ± 0.49	1.20 ± 0.00	1.50 ± 0.71	2.05 ± 0.64	1.25 ± 0.49	1.95 ± 0.64	1.05 ± 0.07	1.00 ± 0.00	0.95 ± 0.07	2.45 ± 0.07	1.85 ± 0.21	1.55 ± 0.07	1.70 ± 0.00
Mandarin EO	0.00 ± 0.00	0.45 ± 0.64	0.45 ± 0.64	1.00 ± 0.28	0.45 ± 0.64	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.45 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.05 ± 0.07	0.50 ± 0.71	0.00 ± 0.00
Cinnamon bark EO	4.95 ± 0.35	5.10 ± 0.42	5.00 ± 1.27	4.50 ± 0.71	3.85 ± 0.21	4.75 ± 1.06	1.80 ± 0.28	2.25 ± 0.21	2.20 ± 0.28	2.20 ± 1.13	3.95 ± 2.90	2.65 ± 1.91	3.50 ± 2.12	2.15 ± 0.07	2.00 ± 0.71	3.50 ± 0.57	3.40 ± 0.42	3.25 ± 0.78	2.70 ± 0.42	2.70 ± 0.42
Orange EO	1.05 ± 0.35	0.90 ± 0.28	1.05 ± 0.35	1.45 ± 0.49	0.45 ± 0.64	0.90 ± 0.14	1.50 ± 0.14	1.35 ± 0.49	0.85 ± 0.35	0.90 ± 0.14	1.15 ± 0.07	0.40 ± 0.57	1.00 ± 0.49	1.15 ± 0.07	1.35 ± 0.21	1.35 ± 0.21	0.00 ± 0.00	0.45 ± 0.64	0.00 ± 0.00	0.30 ± 0.42
Lemon EO	1.15 ± 0.49	0.75 ± 1.06	0.60 ± 0.85	1.00 ± 0.42	0.35 ± 0.49	0.80 ± 0.14	0.00 ± 0.00	1.20 ± 0.14	1.15 ± 0.07	0.00 ± 0.00	1.10 ± 0.14	0.00 ± 0.00	0.45 ± 0.64	0.60 ± 0.85	0.00 ± 0.00	0.00 ± 0.00	0.75 ± 0.07	0.50 ± 0.71	0.00 ± 0.00	0.00 ± 0.00
Clove EO	3.00 ± 0.00	2.75 ± 0.21	2.85 ± 0.35	3.10 ± 0.14	2.55 ± 0.07	3.00 ± 0.00	1.15 ± 0.21	1.15 ± 0.21	1.40 ± 0.57	1.55 ± 0.64	2.25 ± 0.77	1.70 ± 0.71	1.65 ± 0.35	1.45 ± 0.35	1.20 ± 0.42	1.20 ± 0.14	1.30 ± 0.14	1.60 ± 0.42	1.30 ± 0.14	1.35 ± 0.21
Cumin seed EO	3.40 ± 2.97	2.00 ± 0.71	4.25 ± 3.89	6.50 ± 0.71	4.60 ± 4.81	3.45 ± 1.48	1.20 ± 0.00	1.50 ± 0.00	1.00 ± 0.00	1.35 ± 0.35	1.70 ± 0.42	1.05 ± 0.21	0.95 ± 0.21	1.50 ± 0.28	1.25 ± 0.07	1.00 ± 0.00	1.45 ± 0.07	1.00 ± 0.14	0.75 ± 0.07	0.85 ± 0.07
Garlic EO	8.50 ± 0.0	8.50 ± 0.00	8.50 ± 0.00	8.50 ± 0.00	8.50 ± 0.00	8.50 ± 0.00	0.50 ± 0.71	0.40 ± 0.57	1.20 ± 0.57	0.75 ± 0.07	1.35 ± 0.49	0.70 ± 0.99	1.15 ± 0.14	0.00 ± 0.00	0.95 ± 0.07	0.75 ± 0.21	1.65 ± 0.64	1.00 ± 0.28	0.95 ± 0.07	0.90 ± 0.28
Thyme EO	5.75 ± 1.77	5.75 ± 0.35	4.95 ± 0.64	6.00 ± 0.00	4.50 ± 0.00	4.80 ± 0.00	3.25 ± 0.92	3.40 ± 0.57	3.15 ± 0.49	3.70 ± 0.42	5.85 ± 3.04	3.85 ± 1.63	4.60 ± 1.63	3.85 ± 0.21	3.15 ± 0.21	2.65 ± 0.92	4.80 ± 0.14	3.90 ± 0.71	3.70 ± 0.42	4.30 ± 0.28
Rosemary EO	0.85 ± 0.35	0.90 ± 0.28	0.95 ± 0.35	0.85 ± 0.21	0.95 ± 0.07	1.00 ± 0.00	1.35 ± 0.07	1.75 ± 0.07	1.10 ± 0.14	0.95 ± 0.07	1.00 ± 0.00	0.85 ± 0.07	0.95 ± 0.14	1.30 ± 0.42	1.20 ± 0.42	1.00 ± 0.00	0.90 ± 0.00	0.95 ± 0.07	0.40 ± 0.57	0.40 ± 0.57
Sage EO	0.85 ± 0.21	0.95 ± 0.07	1.05 ± 0.35	0.95 ± 0.21	1.30 ± 0.28	1.25 ± 0.35	1.15 ± 0.07	1.35 ± 0.07	0.90 ± 0.14	1.55 ± 0.92	1.75 ± 0.07	1.30 ± 0.28	1.45 ± 0.35	1.50 ± 0.42	1.10 ± 0.28	1.00 ± 0.00	0.90 ± 0.00	1.15 ± 0.78	0.70 ± 0.14	0.90 ± 0.14
Cardamom EO	0.65 ± 0.07	0.85 ± 0.21	0.95 ± 0.21	0.70 ± 0.14	0.95 ± 0.07	0.95 ± 0.07	1.20 ± 0.14	1.80 ± 0.57	1.25 ± 0.35	1.35 ± 0.49	1.50 ± 0.00	1.45 ± 0.21	1.50 ± 0.21	1.35 ± 0.49	1.40 ± 0.57	1.05 ± 0.07	0.90 ± 0.00	0.75 ± 0.07	0.85 ± 0.07	0.85 ± 0.07
Basil EO	1.00 ± 0.57	0.95 ± 0.07	1.30 ± 0.28	0.90 ± 0.14	2.50 ± 2.12	1.80 ± 0.85	0.95 ± 0.07	0.95 ± 0.07	0.75 ± 0.07	1.05 ± 0.21	1.30 ± 0.14	1.15 ± 0.49	0.70 ± 0.28	1.00 ± 0.00	1.05 ± 0.07	1.00 ± 0.00	0.85 ± 0.07	0.75 ± 0.21	0.35 ± 0.49	0.75 ± 0.07
Black pepper EO	0.00 ± 0.00	0.00 ± 0.00	0.35 ± 0.49	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.15 ± 0.07	1.40 ± 0.14	1.05 ± 0.07	1.50 ± 0.71	1.55 ± 0.21	1.45 ± 0.49	1.50 ± 0.64	1.10 ± 0.00	1.35 ± 0.35	1.00 ± 0.00	0.00 ± 0.00	0.45 ± 0.64	0.00 ± 0.00	0.00 ± 0.00
Oleacein	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.40 ± 0.57	0.35 ± 0.49	0.70 ± 0.00	0.85 ± 0.21	0.85 ± 0.07	0.75 ± 0.07	0.75 ± 0.07	1.00 ± 0.28	0.90 ± 0.07	0.40 ± 0.57	1.10 ± 0.14	0.75 ± 0.07	0.75 ± 0.07	0.40 ± 0.57	0.00 ± 0.00	0.00 ± 0.00
Oleokantal	0.00 ± 0.00	0.35 ± 0.49	0.00 ± 0.00	0.35 ± 0.49	0.30 ± 0.42	0.30 ± 0.42	0.90 ± 0.14	1.00 ± 0.14	0.50 ± 0.71	0.85 ± 0.07	0.75 ± 0.07	0.55 ± 0.78	0.90 ± 0.21	0.40 ± 0.57	1.00 ± 0.00	0.60 ± 0.85	0.75 ± 0.07	0.40 ± 0.57	0.00 ± 0.00	0.00 ± 0.00
Nootkatone	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.45 ± 0.64	0.00 ± 0.00	0.00 ± 0.00	1.75 ± 0.49	2.00 ± 0.71	1.45 ± 0.92	2.00 ± 0.00	1.70 ± 0.14	0.55 ± 0.78	1.00 ± 0.00	0.95 ± 0.21	0.95 ± 0.21	1.00 ± 0.14	0.75 ± 0.07	0.85 ± 0.07	0.90 ± 0.42	0.65 ± 0.07

EO, essential oil; *Diameters (cm) of growth inhibition zones expressed as the mean value of duplicate assay for each strain ±standard deviation. Means followed by different letters within each column (a, b, c …) indicate significant differences ($P < 0.05$) between EOs for each strain.

Table 3
Minimum inhibitory concentration (MIC) values of thyme essential oil.

Strain	MIC (μL/mL)
Listeria innocua LI1	1.95 ± 1.85
Listeria innocua LI2	1.95 ± 0.84
Listeria innocua LI3	0.98 ± 0.43
Staphylococcus xylosus	0.98 ± 0.43
Lactobacillus sakei P8-1	3.91 ± 2.02
Lactobacillus sakei SP1-3	3.91 ± 3.87
Lactobacillus curvatus SP7-4	3.91 ± 2.02
Lactobacillus. curvatus SP2-3	3.91 ± 1.69
Debaryomyces hansenii 92	3.91 ± 3.50
Debaryomyces hansenii 94	3.91 ± 3.66

The results are expressed as means ± standard deviation of two independent measurements.

Table 4
Viable counts of *Listeria innocua* (Log cfu/g) in the minced meat samples with no added fat or added with 40% fat and stored at two different temperatures as described in Fig. 1.

	Sampling time points (days)	Storage temperature 1		Storage temperature 2	
		L	L + T	L	L + T
No added fat	**t0**	$7.28 \pm 0.00^{a,B}$	$7.25 \pm 0.08^{a,B}$	$7.28 \pm 0.00^{a,C}$	$7.25 \pm 0.08^{a,C}$
	t3	$6.82 \pm 0.01^{a,C}$	$6.87 \pm 0.04^{a,C}$	$8.64 \pm 0.01^{b,B}$	$9.03 \pm 0.01^{a,B}$
	t6	$7.02 \pm 0.10^{a,BC}$	$7.00 \pm 0.04^{a,BC}$	$9.20 \pm 0.11^{a,A}$	$9.13 \pm 0.00^{a,B}$
	t10	$7.11 \pm 0.01^{a,BC}$	$7.01 \pm 0.01^{b,BC}$	$9.12 \pm 0.10^{a,A}$	$9.38 \pm 0.04^{a,A}$
	t20	$7.96 \pm 0.15^{a,A}$	$7.87 \pm 0.10^{a,A}$	$9.08 \pm 0.04^{b,A}$	$9.19 \pm 0.01^{a,B}$
Fat (40%)	**t0**	$7.12 \pm 0.01^{a,A}$	$6.92 \pm 0.01^{b,B}$	$7.12 \pm 0.01^{a,E}$	$6.92 \pm 0.01^{b,E}$
	t3	$6.93 \pm 0.08^{a,B}$	$6.88 \pm 0.07^{a,B}$	$7.65 \pm 0.05^{a,D}$	$7.82 \pm 0.04^{a,D}$
	t6	$6.74 \pm 0.02^{a,B}$	$6.51 \pm 0.05^{b,C}$	$7.86 \pm 0.06^{a,C}$	$8.07 \pm 0.07^{a,C}$
	t10	$6.76 \pm 0.06^{a,B}$	$6.67 \pm 0.03^{a,C}$	$8.22 \pm 0.01^{b,B}$	$8.37 \pm 0.01^{a,B}$
	t20	$7.31 \pm 0.04^{a,A}$	$7.27 \pm 0.05^{a,A}$	$8.56 \pm 0.04^{a,A}$	$8.65 \pm 0.01^{a,A}$

L, samples inoculated with *Listeria innocua*; L + T, samples inoculated with *L. innocua* and treated with thyme essential oil; Storage temperature 1, 20 °C followed by daily temperature decrease (1 °C) until reaching 14 °C, upheld until the end of 20 days trial; Storage temperature 2, 4 °C for 6 days followed by 10 °C until the end of 20 days trial.
Mean values ± standard deviations of double experiments are shown. Means followed by different letters within each row (a, b, c) and within each column (A, B, C …) indicate significant differences ($P < 0.05$) among different treatments (C, L, L + T) and sampling time points (t) respectively.

our study, no significant differences in the antilisterial activity of thyme EO between samples with 40% fat addition and those without fat addition were observed.

Furthermore, prior research suggests that the lower pH levels of food matrices may make EOs more hydrophobic, which would make it easier for them to pass through the lipid portion of bacterial membranes and, as a result, have stronger antibacterial effects (Yousefi et al., 2020). In the present study, the range of pH values varied between 5.24 and 6.34 for the samples stored at storage temperature 1, and from 5.15 to 7.55 for the samples stored at storage temperature 2 (Table 5). The pH of the fresh minced meat samples in our study is consistent with the pH values previously reported for pork meat (Iacumin & Carballo, 2016). Overall, pH values increased during the storage period, but no clear correlation between pH values and the antilisterial effect of thyme EO was observed.

Concerning a_w, the measured values ranged from 0.8701 to 0.9908 (Table 6). Generally, the a_w increased at the end of the experiment for samples without added fat, while samples with 40% fat exhibited the opposite trend, displaying the lowest a_w values at the final sampling point (t20). Considering that species from the *Listeria* genus are acknowledged for their good tolerance to osmotic stress and high salt concentrations (up to 10% NaCl), they can proliferate in various food matrices characterized by low a_w (≥0.90) (Wiktorczyk-Kapischke et al., 2021). Therefore, as expected, even the lowest a_w values (<0.90) detected at t20 in samples with 40% fat did not negatively affect the growth of *L. innocua*. Furthermore, it has been observed that the antimicrobial activity of EOs might be compromised in food matrices with low a_w and high viscosity. This is because these conditions can reduce the diffusion rate of EOs to their site of action (Cava et al., 2007).

Contrary to the findings of our study, most previous studies have reported significant inhibitory effects of thyme EO against *Listeria* in meat-based products. Aureli et al. (1992) observed that the inclusion of thyme EO in intentionally contaminated minced pork meat samples resulted in a significant decrease in viable *L. monocytogenes* cells during the first week of storage. Furthermore, a bacteriostatic effect of thyme EO against *L. monocytogenes* in beef meatballs stored at 4 °C (Pesavento et al., 2015), as well as against various pathogenic bacteria in irradiated minced meat stored at 5 °C (Barbosa et al., 2009), was reported. Additionally, Amariei et al. (2016) demonstrated that various essential oils, including thyme EO, improved the microbiological stability of minced meat.

The present study revealed minimal or no inhibitory *in vivo* activity of thyme EO against *L. innocua*, which may be due to the low concentration utilized. This suggests the need for higher EO concentrations to achieve satisfactory antimicrobial activity in the *Ciauscolo*-model salami. However, it's important to note that the strong aroma of EOs at higher concentrations raises sensory concerns, thereby limiting their practical utility (Osimani et al., 2023; Posgay et al., 2022). To overcome these limitations, several methods have been suggested. These include encapsulating EOs, using new packaging techniques, incorporating EOs into edible films and coatings, combining them with other preservatives, and adopting emerging technologies like irradiation, high intensity pulsed electric field, high hydrostatic pressure, and cold plasma. These methods aim not only to enhance the effectiveness of EOs in inhibiting the growth of *L. monocytogenes* but also to mask any undesirable sensory characteristics of meat-based products (Osimani et al., 2023; Posgay et al., 2022; Sousa et al., 2022; Yousefi et al., 2020).

However, it is important to assess the drawbacks and cost-effectiveness of using these technologies in salami production. While they show potential for improving product safety and quality, their practical implementation must be carefully evaluated to prevent added complexities and costs (Posgay et al., 2022; Sousa et al., 2022; Yousefi et al., 2020).

4. Conclusions

In our study, among the different tested antimicrobial agents, thyme EO demonstrated the highest efficacy against *L. innocua* in *in vitro* assays. Unexpectedly, our results revealed minimal to no antimicrobial effect of thyme EO against *L. innocua in vivo*, as observed in deliberately contaminated minced pork meat samples stored under temperature conditions typical for the production of Italian PGI *Ciauscolo* salami, selected as a model due to the higher risk of *Listeria* contamination. Furthermore, the fat content of the samples did not notably impact the antimicrobial efficiency of thyme EO.

Our study provides valuable insights for the food industry in two ways. Firstly, the demonstrated effectiveness of EOs *in vitro* suggests their potential as natural biocides for sanitizing surfaces in contact with foods, preventing the formation of *L. monocytogenes* biofilm. Secondly, these findings underscore the challenges encountered in translating *in vitro* findings to practical applications due to the complex interactions between antimicrobial agents and food matrices. Further investigation is needed to understand the factors affecting EO efficacy in real food

Table 5
pH values of the minced meat samples with no added fat or added with 40% fat and stored at two different temperatures as described in Fig. 1.

	Sampling time points (days)	Storage temperature 1			Storage temperature 2		
		C	L	L + T	C	L	L + T
No added fat	**t0**	$5.50 \pm 0.01^{a,C}$	$5.43 \pm 0.03^{a,C}$	$5.49 \pm 0.01^{a,C}$	$5.50 \pm 0.01^{a,D}$	$5.43 \pm 0.03^{a,D}$	$5.49 \pm 0.01^{a,D}$
	t3	$5.61 \pm 0.01^{a,B}$	$5.58 \pm 0.01^{a,B}$	$5.60 \pm 0.01^{a,AB}$	$5.53 \pm 0.01^{a,D}$	$5.46 \pm 0.01^{b,D}$	$5.54 \pm 0.02^{a,CD}$
	t6	$5.59 \pm 0.01^{a,B}$	$5.59 \pm 0.01^{a,B}$	$5.63 \pm 0.02^{a,A}$	$5.59 \pm 0.01^{a,C}$	$5.54 \pm 0.01^{a,C}$	$5.60 \pm 0.03^{a,C}$
	t10	$5.51 \pm 0.01^{b,C}$	$5.53 \pm 0.01^{ab,B}$	$5.57 \pm 0.01^{a,B}$	$6.19 \pm 0.01^{b,B}$	$6.29 \pm 0.01^{a,B}$	$6.17 \pm 0.01^{b,B}$
	t20	$6.30 \pm 0.01^{a,A}$	$6.34 \pm 0.01^{a,A}$	$5.62 \pm 0.01^{b,AB}$	$7.55 \pm 0.01^{a,A}$	$7.39 \pm 0.01^{b,A}$	$7.06 \pm 0.01^{c,A}$
Fat (40%)	**t0**	$5.30 \pm 0.01^{a,B}$	$5.30 \pm 0.01^{a,B}$	$5.30 \pm 0.01^{a,D}$	$5.30 \pm 0.01^{a,B}$	$5.30 \pm 0.01^{a,C}$	$5.30 \pm 0.01^{a,C}$
	t3	$5.24 \pm 0.01^{c,B}$	$5.36 \pm 0.03^{b,B}$	$5.45 \pm 0.01^{a,B}$	$5.15 \pm 0.01^{b,C}$	$5.43 \pm 0.02^{a,B}$	$5.46 \pm 0.01^{a,B}$
	t6	$5.61 \pm 0.03^{a,A}$	$5.46 \pm 0.01^{b,A}$	$5.36 \pm 0.03^{b,CD}$	$5.42 \pm 0.01^{a,A}$	$5.38 \pm 0.01^{ab,B}$	$5.32 \pm 0.03^{b,C}$
	t10	$5.38 \pm 0.01^{b,B}$	$5.49 \pm 0.03^{a,A}$	$5.38 \pm 0.01^{b,C}$	$5.28 \pm 0.01^{b,B}$	$5.47 \pm 0.03^{a,A}$	$5.39 \pm 0.01^{a,B}$
	t20	$5.63 \pm 0.09^{a,A}$	$5.36 \pm 0.01^{b,B}$	$5.66 \pm 0.01^{a,A}$	$5.28 \pm 0.01^{c,B}$	$5.46 \pm 0.01^{b,A}$	$5.54 \pm 0.01^{a,A}$

C, control (untreated) samples; L, samples inoculated with *Listeria innocua*; L + T, samples inoculated with *L.innocua* and treated with thyme essential oil; Storage temperature 1, 20 °C followed by daily temperature decrease (1 °C) until reaching 14 °C, upheld until the end of 20 days trial; Storage temperature 2, 4 °C for 6 days followed by 10 °C until the end of 20 days trial.
Mean values ± standard deviations of double experiments are shown. Means followed by different letters within each row (a, b, c) and within each column (A, B, C …) indicate significant differences ($P < 0.05$) among different treatments (C, L, L + T) and sampling time points (t) respectively.

Table 6
Water activity of the minced meat samples with no added fat or added with 40% fat and stored at two different temperatures as described in Fig. 1.

	Sampling time points (days)	Storage temperature 1			Storage temperature 2		
		C	L	L + T	C	L	L + T
No added fat	**t0**	$0.9713 \pm 0.0004^{a,C}$	$0.9692 \pm 0.0001^{b,D}$	$0.9663 \pm 0.0001^{c,E}$	$0.9713 \pm 0.0004^{a,C}$	$0.9692 \pm 0.0001^{b,C}$	$0.9663 \pm 0.0001^{c,B}$
	t3	$0.9825 \pm 0.0002^{b,AB}$	$0.9782 \pm 0.0002^{a,C}$	$0.9818 \pm 0.0004^{a,C}$	$0.9796 \pm 0.0003^{b,B}$	$0.9868 \pm 0.0008^{a,A}$	$0.9849 \pm 0.0010^{a,A}$
	t6	$0.9595 \pm 0.0001^{c,D}$	$0.9663 \pm 0.0004^{b,E}$	$0.9686 \pm 0.0004^{a,D}$	$0.9694 \pm 0.0003^{a,C}$	$0.9606 \pm 0.0008^{b,D}$	$0.9687 \pm 0.0008^{a,B}$
	t10	$0.9813 \pm 0.0004^{b,B}$	$0.9854 \pm 0.0007^{a,B}$	$0.9836 \pm 0.0005^{a,B}$	$0.9840 \pm 0.0007^{a,A}$	$0.9847 \pm 0.0002^{a,AB}$	$0.9845 \pm 0.0006^{a,A}$
	t20	$0.9841 \pm 0.0007^{c,A}$	$0.9908 \pm 0.0010^{a,A}$	$0.9876 \pm 0.0002^{b,A}$	$0.9707 \pm 0.0008^{b,C}$	$0.9842 \pm 0.0002^{a,B}$	$0.9836 \pm 0.0008^{a,A}$
Fat (40%)	**t0**	$0.9067 \pm 0.0001^{c,D}$	$0.9145 \pm 0.0001^{b,D}$	$0.9332 \pm 0.0001^{a,D}$	$0.9067 \pm 0.0001^{c,D}$	$0.9145 \pm 0.0001^{b,D}$	$0.9332 \pm 0.0001^{a,D}$
	t3	$0.9484 \pm 0.0002^{a,A}$	$0.9348 \pm 0.0004^{c,C}$	$0.9383 \pm 0.0004^{b,B}$	$0.9454 \pm 0.0003^{a,B}$	$0.9365 \pm 0.0005^{b,C}$	$0.9348 \pm 0.0004^{b,C}$
	t6	$0.9444 \pm 0.0002^{a,B}$	$0.9397 \pm 0.0002^{b,B}$	$0.9359 \pm 0.0005^{c,C}$	$0.9302 \pm 0.0001^{c,C}$	$0.9392 \pm 0.0001^{b,B}$	$0.9429 \pm 0.0005^{a,B}$
	t10	$0.9225 \pm 0.0001^{b,C}$	$0.9461 \pm 0.0001^{a,A}$	$0.9465 \pm 0.0001^{a,A}$	$0.9607 \pm 0.0003^{a,A}$	$0.9486 \pm 0.0004^{b,A}$	$0.9497 \pm 0.0003^{b,A}$
	t20	$0.8701 \pm 0.0001^{c,E}$	$0.8764 \pm 0.0002^{b,E}$	$0.8786 \pm 0.0004^{a,E}$	$0.8921 \pm 0.0008^{a,E}$	$0.8784 \pm 0.0004^{c,E}$	$0.8864 \pm 0.0003^{b,E}$

C, control (untreated) samples; L, samples inoculated with *Listeria innocua*; L + T, samples inoculated with *L.innocua* and treated with thyme essential oil; Storage temperature 1, 20 °C followed by daily temperature decrease (1 °C) until reaching 14 °C, upheld until the end of 20 days trial; Storage temperature 2, 4 °C for 6 days followed by 10 °C until the end of 20 days trial.
Mean values ± standard deviations of double experiments are shown. Means followed by different letters within each row (a, b, c) and within each column (A, B, C …) indicate significant differences ($P < 0.05$) among different treatments (C, L, L + T) and sampling time points (t) respectively.

systems. Our observations indicate that higher EO concentrations may be needed for satisfactory antimicrobial activity, but their strong aroma raises sensory concerns. Various promising approaches, like encapsulation and innovative packaging, offer potential to enhance EO efficacy against *Listeria* in processed meats while addressing sensory issues. However, a thorough assessment of their drawbacks and cost effectiveness is crucial for practical implementation in real food systems.

CRediT authorship contribution statement

Vesna Milanović: Writing – original draft, Formal analysis, Conceptualization. **Mariana Mariz:** Investigation, Formal analysis. **Federica Cardinali:** Writing – original draft, Investigation, Formal analysis. **Cristiana Garofalo:** Resources, Formal analysis. **Mila Radan:** Formal analysis. **Tea Bilušić:** Formal analysis. **Lucia Aquilanti:** Writing – review & editing, Resources, Formal analysis. **Luís Miguel Cunha:** Writing – review & editing, Resources. **Andrea Osimani:** Writing – review & editing, Supervision, Data curation, Conceptualization.

Declaration of competing interest

Regarding the manuscript titled "***Evaluation of natural compounds against Listeria innocua: Translating in vitro success to processed meat models***" submitted for publication in Food Bioscience, the authors declare that NO conflict of interest exists.

Data availability

Data will be made available on request.

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