

Evaluation of Antibacterial Properties of Commercial Essential Oils on Foodborne Pathogens in a Liver Pâté-Type Product

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In the past, several efforts have been made to find suitable substitutes for synthetic preservatives in meat products with fewer side effects on human health. Essential oils have gained worldwide interest due to their antimicrobial activity. The addition of these aromatic compounds to foods may be hampered by their strong sensory characteristics (taste and smell). The objective of this study was to evaluate the efficiency of five essential oils (EOs) (*Ocimum basilicum* L., *Origanum vulgare* L., *Rosmarinus officinalis* L., *Salvia officinalis* L., and *Thymus vulgaris* L.) against important foodborne pathogens. First, a microdilution assay was carried out to determine the minimal inhibitory concentration (MIC) of the EOs against *Staphylococcus (Staph.) aureus* ATCC 6538, *Salmonella (S.) enterica* subsp. *enterica* serovar Typhimurium ATCC 14028, and *Escherichia (E.) coli* ATCC 25922. Since the EOs inhibited the growth of these bacteria, their activity was studied in a real food matrix. The *in vivo* test was performed on the model of liver pâté: the homogenized and heat-treated samples were formulated with EOs at concentrations of 1 MIC and 2 MIC, inoculated with bacterial suspensions (10^5 CFU/g), packaged under vacuum, and stored at 4 °C for 3 days. The addition of 2 MIC of thyme and sage oils showed a significant reduction in the viable counts of *E. coli*, *S. Typhimurium*, and *Staph. aureus* compared to the control samples.

Overall, this study demonstrated that thyme and sage EOs, as natural preservatives, had great potential to prevent the growth of important foodborne pathogens (*E. coli*, *S. Typhimurium*, and *Staph. aureus*) in liver pâté, but their efficiency was highly dose-dependent. However, the tested concentrations of EOs (1 MIC and 2 MIC) had an influence on the sensory characteristics of the finished products that may hinder their future applicability to improve the shelf-life of meat products. Therefore, further studies are required to clarify such an issue.

1. Introduction

Due to globalization and the active food trade, foodborne diseases caused by various bacteria, fungi, viruses, and parasites have become a leading health problem worldwide (Lee and Yoon, 2021). According to the World Health Organization (2020), these microbes cause around 600 million cases of foodborne infections and 450,000 deaths every year, and these numbers are expected to increase in the future (Lee and Yoon, 2021). While most cases of foodborne illnesses are self-limiting (Rivera et al., 2018), they can cause long-term health complications or even result in death in susceptible populations (Lund, 2019).

Pork is one of the most commonly consumed meat (Razmaitė et al., 2022). Porcine liver pâté is a traditional fat product (Estévez et al., 2004) made of minced liver, meat, and fat mixed with water and additives (Agregán et al., 2018). Although the meat and offal of healthy animals are almost free of microorganisms, they are perishable raw materials (Bantawa et al., 2018) that may provide an ideal environment for both spoilage and pathogenic bacteria (e.g., *Salmonella* spp, *Staphylococcus aureus*, *Escherichia coli*, etc.) (Boskovic et al., 2015). These microbes can contaminate fresh meat (Favaro and Todorov, 2017) from various sources at any stage of production, including the environment, gastrointestinal tract and skin surfaces of animals, and used equipment (Abdel-Sater et al., 2017).

Most liver pâté-type products are prepared with the addition of sodium nitrite to prevent the proliferation of undesirable microflora (Lucas-González et al., 2021). However, the consumer demand for minimally processed foods without synthetic additives has been continuously growing (Kocić-Tanackov et al., 2020). In order to ensure the chemical and microbiological safety of foods, essential oils (EOs) derived from medicinal and aromatic plants have emerged as an ideal substitute for artificial preservatives due to their strong antimicrobial activity and lower health concerns. The application of these naturally occurring compounds may be an effective way to reduce spoilage (Tajkarimi et al., 2010), inhibit the growth of foodborne pathogens, and increase consumer acceptance (Yu et al., 2021). Unlike antibiotics, volatiles has certain advantages since pathogenic microorganisms may not develop resistance against them as their constituents show a great diversity in their mechanisms of action (Baldim et al., 2018) (e.g., affecting cellular respiration, energy metabolism, DNA, cell wall, or cell membrane, etc.); (Ju et al., 2018). To improve the applicability and effectiveness of these bio-preservatives, EOs may be combined with other hurdles, including other antimicrobials, low temperature, modified atmosphere packaging, high-hydrostatic pressure, low dose irradiation (Posgay et al., 2022), active packaging (Torrieri et al., 2015), or edible coatings (Nga et al., 2022)

In the past years, EOs derived from *Ocimum* (Gaio et al., 2015), *Origanum* (Busatta et al., 2007), *Salvia*, and *Thymus* (Boskovic et al., 2017) species have been examined widely and applied successfully as antimicrobial and antioxidant agent in various meat products including minced meats, sausages, and pâtés as well (Moraes-Lovison et al., 2017). These EOs are rich sources of compounds, such as carvacrol (Laroque et al., 2023), thymol (Tian et al., 2021), linalool (Liu et al., 2020), α - and β -thujone, or camphor (Danilović et al., 2021) that are associated with strong bioactivity. However, the effectiveness of EOs may be unpredictable depending on several factors (EO composition and concentration, chemical composition of the food matrices) (Posgay et al., 2022).

Therefore, the aims of this study were to determine (1) the minimal inhibitory concentration of commercially available EOs (*Ocimum basilicum*, *Origanum vulgare*, *Rosmarinus officinalis*, *Salvia officinalis*, *Thymus vulgaris*) and (2) examine their *in vivo* efficiency in a liver pâté-type matrix. To the authors' knowledge, this is the first study that has used EOs derived from basil, oregano, salvia, and thyme to control the growth of potential pathogenic bacteria in pork liver pâté.

2. Materials and methods

2.1 Materials

The pork liver and fat used for the experiments were obtained from a nearby meat processing plant (Darnó-Hús, Funkció Ltd., Darnózséli, Hungary). Samples of essential oils (EOs) were purchased online from NHR Organic Oils Ltd. (Brighton, UK) and NSH Organics Ltd. (Budapest, Hungary). The major constituents of the applied EOs are listed in Table 1. The used bacterial strains (*Staphylococcus aureus* ATCC 6538, *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 17028, and *Escherichia coli* ATCC 25922) were cultured and maintained on Tryptic soy agar plates (TSA; Biolab, Budapest, Hungary).

Table 1. List of applied essential oils purchased from different suppliers.

Botanical species	Family	Country of collection	Part of herb used	Major constituents	Distributor
<i>Salvia officinalis</i> L.	Lamiaceae	Albania	Flower	α -thujone, camphor, limonene, β -thujone	NHR Organic Oils Ltd. (UK)
<i>Rosmarinus officinalis</i> L.	Lamiaceae	Tunisia	Flower	1,8-cineole, α -pinene, camphor, β -pinene	
<i>Origanum vulgare</i> L.	Lamiaceae	Hungary	Flower and leaves	Carvacrol, <i>p</i> -cymene, γ -terpinene	
<i>Ocimum basilicum</i> L.	Lamiaceae	Hungary	Leaves	Linalool, 1,8-cineole, eugenol	NSH Organics Ltd. (Hungary)
<i>Thymus vulgaris</i> L.	Lamiaceae	NA	NA	Thymol, <i>p</i> -cymene, γ -terpinene, carvacrol, linalool	

2.2 Manufacture the liver pâté

The liver pâtés were produced at the Department of Food Science, Albert Kázmér Agricultural Faculty, Széchenyi István University. First, the liver was cooked to a core temperature of 81 °C and de-heated for 1 h, so that the effect of essential oils on the added bacteria could be tested, presumably in a sterile product. The

product was chopped in a cutter (Nagama VVB, Dresden, Germany), and fat was added to a level of 25 % of the fat content of the final product for ease of handling.

The product was loaded into collagen casings (Böllér-Ker Ltd., Pápa, Hungary), vacuum packaged in boilable vacuum bags, and cooked at 81 °C core heat for 1 h. The finished product was stored in a refrigerator at approximately 4 °C until further analysis.

The uninoculated samples were tested for total plate count, coliform count, and the presence or absence of potentially pathogenic bacteria (e.g., *Staphylococcus aureus*, *Clostridium perfringens*, *Enterococcus faecalis*, *Salmonella* spp., and *Escherichia coli*) according to Decree No. 4/1998 (XI. 11.) on the acceptable levels of microbiological contamination in foods (Ministry of Health, 1998).

2.3 Determination of the minimum inhibitory concentration (MIC) of essential oils

The MIC of EOs was determined by using the macrodilution method recommended by the Clinical and Laboratory Standards Institute guidelines (CLSI, 2012). The EOs were dissolved in 5 % of dimethyl sulfoxide solution (Molar Chemicals, Halásztelek, Hungary) containing 0.1 % of TWEEN-80 (Biolab Zrt, Budapest, Hungary) (Cazella et al., 2019) and two-fold serially diluted with cation-adjusted Mueller-Hinton broth (Biolab Zrt, Budapest, Hungary) to obtain the final concentrations from 0.39 to 200 µl/mL (Perdana et al, 2021). *Staphylococcus aureus* ATCC 6538, *Salmonella enterica subspecies enterica serovar* Typhimurium ATCC 17028, and *Escherichia coli* ATCC 25922 bacterial suspensions were added to the tubes (5×10^5 CFU/mL) and mixed thoroughly. After incubation (37 °C for 16 h). 100 µL resazurin solution (0.01 %) (Germed, German Democratic Republic) was added to each tube and further incubated for 1 h at 37 °C. The MIC was defined as the lowest concentration of an EO at which no colour change (visible bacterial growth) was detected in the tubes.

2.4 Antibacterial activity of essential oils in liver pâté samples

To determine the *in vivo* efficacy of EOs, bacterial suspensions of *E. coli*, *S. Typhimurium*, *Staph. aureus* were prepared by adjusting the cell count to 0.5 McFarland standard turbidity and added to the liver pâté mixture to achieve a final concentration of 10^5 CFU/g. To confirm the initial bacterial count, the suspensions were spread-plated on Müller-Hinton agar (Biolab Zrt, Budapest, Hungary) and incubated at 37 °C for 24 h. The inoculated pâté samples were vacuum packaged and cool-stored at 4 °C.

2.5 Microbiological analysis of liver pâté samples

Samples for microbiological analysis were collected after 0, 16, 24, 48, and 72 h. Control products were prepared without EO addition. For the enumeration of pathogens, a 10 g sample was diluted with 90 mL of sterile saline solution (0.85%) and homogenized using a Stomacher 400 Circulator (Seward, Worthing, UK). The homogenate was serially diluted up to 10^{-6} and 0.1 µl/mL of a dilution was spread on the surface of the TSA medium. After incubation (at $37 \pm 2^\circ\text{C}$ for 24 h), plates with 30-300 colonies were counted.

2.6 Statistical analysis

The results presented in this paper are the means \pm SD of three replicate analyses. For the statistical analysis, two-sample t-test was carried out, and statistical significance was set at $p < 0.05$ in all cases.

3. Results and discussion

3.1 The microbiological status of the uninoculated liver pâté samples

As expected, the heat treatment of the samples was satisfactory, as no microbial growth was detected in the uninoculated liver pâté mixture regarding the overall microbiological status (total plate count, coliform count, presence of potential human pathogens) (data not shown).

3.2 Minimum inhibitory concentration (MIC) assay

The results of the MIC determination showed that *E. coli*, *S. Typhimurium*, and *Staph. aureus* was susceptible against the EOs of basil, oregano, sage, and thyme, with MIC values from 0.52 to 5.21 µL/mL. In addition, the Gram-negative bacteria were more sensitive to the EOs. The rosemary oil had only a weak antibacterial effect compared to the other extracts; thus, this EO was not included during the *in vivo* experiments.

Table 2: EO concentrations based on the results of the minimum inhibitory concentration (MIC) used during the first *in vivo* experiment

MICROORGANIS M	EO	MIC ($\mu\text{L/ML}$)
ESCHERICHIA COLI	Thyme	0.52
	Oregano	0.78
SALMONELLA TYPHIMURIUM	Thyme	3.65
	Basil	5.21
STAPHYLOCOCC US AUREUS	Sage	5.21

3.3 Results of the first *in vivo* test using 1 MIC of essential oils

During the first experiment, thyme, oregano, basil, and sage EOs were added to the mixtures at 1 MIC concentrations (Table 2.). Compared to the control sample, no significant differences were found in the products supplemented with EO (data not shown). Overall, the applied volatiles could not hinder the growth of the tested pathogens.

3.4 Results of the second *in vivo* test using 2 MIC of essential oils

As shown in Figure 1., the addition of *T. vulgare* and *O. vulgare* EO at a concentration of 2 MIC inhibited the proliferation of *E. coli* in the liver pâté. The greatest reduction in the bacterial counts was observed in the product containing oregano oil. At 72 h, the difference between the cell counts of the control pâté and samples supplemented with EOs was around 0.6-logs. These results proved that the addition of thyme oil significantly decreased the levels of *E. coli* compared to the control sample.

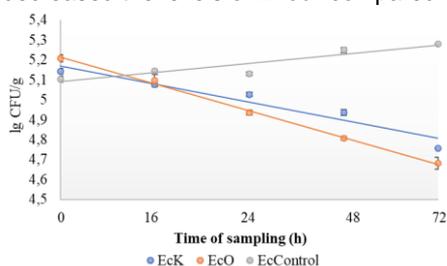


Figure 1: Results of the microbiological analysis of liver pâté inoculated with *Escherichia coli* ATCC 25922 (EcK: product with *E. coli* and thyme oil; EcO: product with *E. coli* and oregano oil; EcControl: control product with *E. coli*)

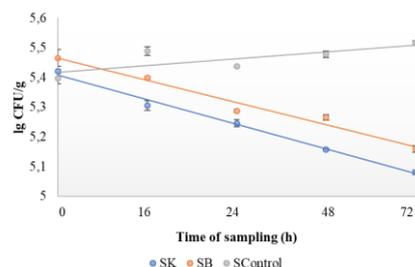


Figure 2: Results of the microbiological analysis of liver pâtés inoculated with *Salmonella enterica* subsp. *enterica* serovar *Typhimurium* ATCC 14028 (SK: product with *S. Typhimurium* and thyme oil; SB: product with *S. Typhimurium* and basil oil; SControl: control product with *S. Typhimurium*)

Similar results were obtained when oregano and thyme EOs were added to liver pâté inoculated with *S. Typhimurium* (Figure 2.). During storage, the cell counts of *Salmonella* declined sharply by at least 0.3 logs in the EO-supplemented products. The control sample could not hinder the growth of this pathogenic bacteria. In this case, the difference between the product containing thyme oil and the control was also significant ($p=0.0216$).

Concerning *Staph. aureus*, sage EO was added to the pâté mixture to control bacterial growth. For the control sample, the cell counts increased until the third (24 h) sampling, after which it started to decrease slightly. On the other hand, the cell counts of the product containing sage oil gradually decreased until the end of the experiment. The difference between the bacterial counts of the control and the essential oil-supplemented products was around 0.4-logs after the 72 h long storage.

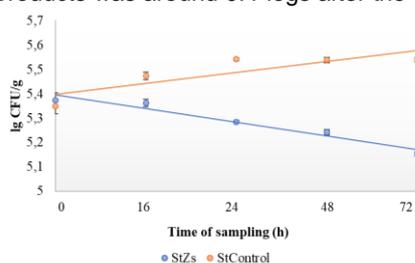


Figure 3: Results of the microbiological analysis of liver pâtés inoculated with *Staphylococcus aureus* ATCC 6538 (StZs: product with *Staph. aureus* and sage oil; StControl: control product with *Staph. aureus*)

4. Conclusions

In this study, commercially available EOs were utilized as bio-preservatives for the production of a pork liver pâté. The MIC values of basil, oregano, sage, and thyme oils showed that these EOs have a strong antibacterial activity against important foodborne pathogens, such as *E. coli*, *S. Typhimurium*, and *Staph. aureus*. Based on the *in vivo* experiments, EOs may be used during the production of various meat-based products to inhibit the growth of microorganisms and extend the shelf-life of these products, but their application is highly dose-dependent due to the complex chemical composition of meat matrices. On the other hand, a higher concentration of these active compounds may have a negative effect on the sensorial characteristics (smell, taste) of the final product and lower consumer acceptance. The combination of EOs with other hurdle technologies may provide an alternative method to overcome these negative effects.

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