

ANTI-LISTERIAL EFFECT OF SELECTED ESSENTIAL OILS AND THYMOL

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The anti-listerial effect of marjoram, thyme essential oils (EOs) and thymol on *Listeria monocytogenes* inoculated chicken breast fillets was investigated. Before inoculation the fillets were pretreated by washing or not under running tap water. Inoculated samples were kept at 6 °C for 24 h to allow the growth of *L. monocytogenes*. After this, the fillets were put in marinating solutions containing salt (5%) and EOs or thymol in MIC/2 concentration established *in vitro*. Total germ count (TGC) and *L. monocytogenes* count was monitored on the meat surface and in the marinating solutions following 24 and 48 h storage at 6 °C. Thyme and thymol reduced significantly *Listeria* cell count (1–3 log CFU) in both samples. They also gave good flavour to the fried meat. The doses of EOs used were optimal for antimicrobial efficiency and had a pleasant flavour effect. Washing was not efficient in reducing total germ count.

Keywords: *Listeria* – essential oils – chicken meat – sensory evaluation

INTRODUCTION

Raw poultry meat products have frequently been found to be contaminated with pathogenic microorganisms which can cause life threatening diseases [12]. *Listeria monocytogenes* is one of the most important food-borne pathogens occurring in wide range of foods such as milk, meat products, fish and seafood [15]. The bacterium is capable of growing at refrigeration temperatures, low pH and in the presence of high salt concentrations making it difficult to control [7]. Immune compromised patients, pregnant women and the elderly are the most sensitive for *Listeria* infections. The ability of this bacterium to adapt efficiently to different environments by forming resistant biofilms on different surfaces makes the problem even more serious. These biofilms could be formed on various equipments and food handling surfaces used in the food industry [2]. The *Listeria* may persist in food plants for months and up to several years [18]. It has been demonstrated that the rapid adhesion of this bacterium to a surface, combined with biotransfer potential throughout the biofilm formation

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stages, makes *L. monocytogenes* a potential risk for the food industry; cells can easily be transferred to food products during processing [4]. Biofilm formation and growth can continue on the surface of foods leading to outbreaks of listeriosis. Because biofilms easily create resistance against a lot of commonly used antimicrobials and preservatives the need for developing new eradication strategies is emerging [8].

There is a growing interest in using essential oils (EOs) as natural preservatives against food spoilage- and food-borne pathogens [3]. EOs with phenolic compounds like thyme, oregano, mint and others are known to possess good antimicrobial properties. They can control the growth of pathogens and can extend shelf life of food products [3, 12].

The food industry primarily uses EOs as flavourings. Their use as food preservatives requires further knowledge about their mode of action and the effect of the food matrix components on their antimicrobial properties [1, 9]. It is known that EOs cause increased membrane permeability due to damage in membrane integrity, which affects pH homeostasis and equilibrium of inorganic ions [9]. Several studies are published regarding the effect of EOs on food borne pathogens like *Listeria*. Targeting surface colonization rather than bacterial fitness seems to be a good approach, because the selective pressure is lower and may lead to fewer resistant strains [16]. In a recent study, raw chicken fillets were inoculated with *L. monocytogenes* and the effects of chitosan (a commonly used preservative) and oregano EO were tested for the extension of shelf life of the meat. Both compounds (especially chitosan) had a significant antibacterial and preservative effect. When these antimicrobials used in combination they extended the shelf life of the chicken fillets with six days [12].

The latest guidelines of United States Department of Agriculture (USDA) and Food Safety Authority (FSA) [6, 20] do not recommend washing of the poultry meat to avoid spreading of *Campylobacter* and other possible pathogens. However, in households washing of the poultry meat before use is a common practice. The aim of the present study was to investigate the anti-listerial effect of marjoram, thyme EOs and thymol on washed and unwashed chicken breast fillets.

MATERIALS AND METHODS

Bacterial strain

The Gram-positive bacterium *Listeria monocytogenes* (strain code: NCAIM B01934) was obtained from the National Collection of Agricultural and Industrial Microorganism (Corvinus University, Budapest, Hungary) and deposited at $-80\text{ }^{\circ}\text{C}$ in the Szeged Microbiological Collection (strain code: SZMC 21307) maintained by the Department of Microbiology, University of Szeged (Szeged, Hungary). Cultivations were carried on Palcam Selective Agar (Lab M, UK) at $37\text{ }^{\circ}\text{C}$.

Essential oils

Based on the results of previous experiments [11, 17] marjoram (*Origanum majorana*) and thyme (*Thymus vulgaris*) EOs were involved in this study. They were purchased from Aromax Natural Products Ltd. (Budapest, Hungary). Thymol (the major component of thyme EO) was obtained from Sigma-Aldrich (Hungary). The compositions of the oils were determined by GC-MS (Agilent 6850 Series II; MS: 5975C VL MSD; Agilent Technologies, Palo Alto, CA, USA) using an Agilent 19091S-433E columna at the laboratory of Aromax Natural Products Ltd. (Budapest, Hungary).

In vitro determination of minimal inhibitory concentration (MIC)

For determination of MIC values the bacterium was grown in Tryptic Soya Broth (TSB; peptone from casein: 17 g (Merck, Hungary), peptone from soy meal: 3 g (Oxoid, UK), D(+)-glucose: 2.5 g (VWR, Hungary), NaCl: 5 g (VWR, Hungary), K₂HPO₄ 2.5 g for 1000 mL) at 37 °C. The dilutions of the EOs were made in liquid culture media in combination with Tween 40 (1%). Thymol was dissolved in ethanol before it was added to liquid culture media. One hundred µl of 24-h-old cell suspension (10⁶ CFU/mL) in liquid culture medium was added to the wells of a 96-well microtiter plate, followed by 100 µl of the diluted EO or the component. Positive controls contained the inoculated growth medium without any EOs or components and negative controls contained EOs or thymol in sterile medium. After 24 h incubation at 37 °C, absorbance was measured at 600 nm (SPECTROstar Nano spectrophotometer, BMG Labtech, Germany). Decrease of the absorbance of the treated samples to 10% or lower of the positive control was considered as the MIC value. Measurements were made in triplicates. Bacteriostatic or bacteriocidal character of inhibition was determined by plating 100 µL suspensions from MIC or higher concentrations onto PALCAM selective medium. The MIC/2 values of these oils and thymol were used for marinating the chicken breast fillets.

Pretreatment inoculation and marinating of chicken breast fillets

Fresh, skinless chicken breast fillets were purchased from a local market (Szeged, Hungary). They were cut aseptically in 1 cm thick 5 × 5 cm cubes. The two sample groups were unwashed and washed meat cubes where the washing was made with running tap water. After pretreatment, the cubes were soaked with sterile paper kitchen towel and placed in sterile polystyrene boxes. Boxes were sterilized by soaking with chlorinated water (1000 ppm) followed by illumination with UV light (30 min) in a biological safety cabinet. Chicken breast cubes were inoculated with bacterial suspension prepared in sterile distilled water from a 24-h-old *L. monocy-*

togenes culture. One hundred μL was pipetted onto the surface of each cube and evenly distributed with a bent glass spreader. Inoculating suspension contained approximately 10^4 CFU corresponding to 10^2 CFUs/cm². Inoculum size was adjusted according to the natural *Listeria* counts determined for raw poultry foodstuffs [14] and the European legislation limit (Regulation (EC) No 2073/2005). Samples were kept for 24 h at refrigerated conditions (6 ± 1 °C) for listerial growth. Three marinating solutions were prepared containing 5% (w/v) salt and one of the EOs or thymol in MIC/2 concentrations. Thymol (purchased in powder form) was first dissolved in 500 μL ethanol (96%, v/v). Thymol solution or the essential oils were directly pipetted onto the solid salt and then they were dissolved in sterile distilled water. Boxes with the two different (washed and unwashed) chicken samples were filled with these marinating solutions covering the whole surface of the meat cubes. Control samples contained only 5% salt without any other seasoning. Samples were incubated for 24 and 48 h at refrigerated conditions (6 ± 1 °C).

Microbiological analysis of chicken breast fillets

For determination of the natural microbial counts of fresh chicken fillets meat surfaces were sampled with sterile culture swabs, which were placed in test tubes containing 9 mL of sterile peptone water. The same sampling method was used after treatments with EOs. Marinating solutions were analyzed by adding 1 mL of sample to 9 mL peptone water. Total germ count (TGC) was determined using Plate Count Agar (Merck, Hungary). Number of coliform bacteria were determined using Brilliant Green Broth. Presence of *Escherichia coli* was determined by inoculating 1 mL from the positive tubes into tryptophan broth to detect indol production. The presence of *Salmonella* was established after pre-enrichment carried out by adding 25 g of meat to 225 mL peptone water. *Staphylococcus aureus* was investigated on Baird Parker Agar (Merck, Hungary) and presence of *Listeria* was determined on Palcam Selective Agar (Merck, Hungary).

Sensory evaluation

Marinated, non-inoculated meat cubes were fried in sunflower oil. A panel of six untrained judges was asked to evaluate odour and taste intensities of the fried meat. The reference sample was kept in a solution containing only 5% salt. Odour and taste acceptability was estimated using a hedonic scale ranging from 5 to 1 with 5 corresponding to the most-liked and 1 corresponding to the least-liked sample. The limit of acceptability was established at 3. Sensory evaluations were repeated twice with different chicken samples.

Statistical analysis

Microbiological data were transformed into logarithms of the number of colony forming units (CFU/cm²) and were subjected to one-way analysis of variance (ANOVA). Statistical analysis was conducted using R Works 2.8.0. Significance was considered at $P < 0.05$.

RESULTS

Composition and in vitro MIC values of EOs

The main components of marjoram and thyme EOs are indicated in Table 1. Marjoram had terpinen-4-ol (terpene alcohol) as main component with considerable amounts of γ -terpinene and cis-sabinene hydrate. Thyme EO had thymol (phenolic compound) as major component and a considerable amount of its precursor p-cymene. MIC values of EOs against *L. monocytogenes* were 3.5 mg/mL for marjoram and 2 mg/mL for thyme. The best inhibitory effect was achieved with thymol (0.5 mg/mL).

Table 1
Chemical composition of essential oils (%), as given by the producer

Components (%)	Marjoram <i>Origanum majorana</i>	Thyme <i>Thymus vulgaris</i>
p-cymene	–	31.7
Terpinen-4-ol	33.5	–
Thymol	–	51.8
β -phellandrene	8	–
γ -terpinene	19.5	6.2

– Not detected.

Microbiological load of chicken fillets before and after inoculation

The initial TGC of the fresh chicken meat purchased from the market was 4.3 log CFU/cm². Coliform numbers were less than 10 CFU/cm² and no *Staphylococcus*, *Salmonella* or *Listeria* was detected. This means that the fillets met the demands of the European and Hungarian food safety standards. After 24-h pre-incubation with *Listeria*, unwashed and washed cubes had 2.9 and 3.4 log CFU/cm², respectively. *E. coli* numbers were less than 10 CFU/cm² and neither *Staphylococcus* nor *Salmonella* could be detected.

*Microbiological changes of inoculated chicken breast fillets
after marinating*

Changes in TGC and *Listeria* counts on unwashed and washed meat cubes are presented in Figures 1 and 2. After 24-h storage significant reduction of TGC was achieved only with thymol (2.5 log reduction, $P < 0.001$). *Listeria* counts dropped considerably following treatment with thyme EO (0.45 log reduction, $P < 0.001$). After 48 h, the TGC values of the control sample increased to 5.15 log CFU/cm², but in

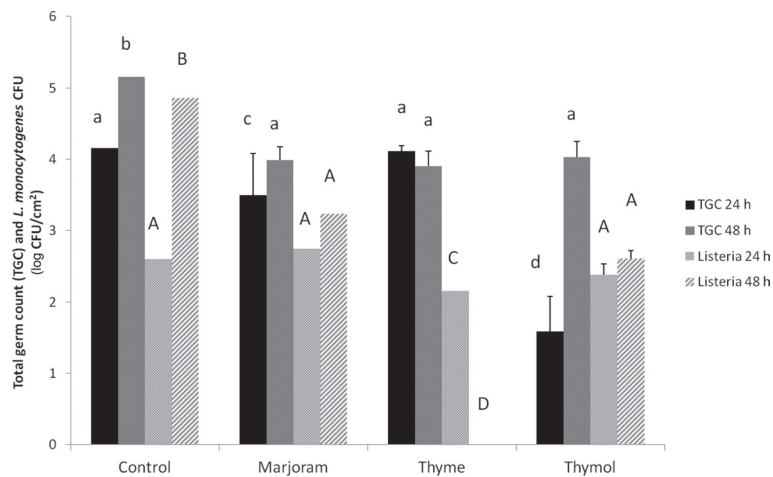


Fig. 1. Total germ counts (TGC) and *Listeria* CFU after marinating of the unwashed samples. Lower cases show comparison between EOs and the control sample in case of TGC and upper cases, comparison between *L. monocytogenes* counts

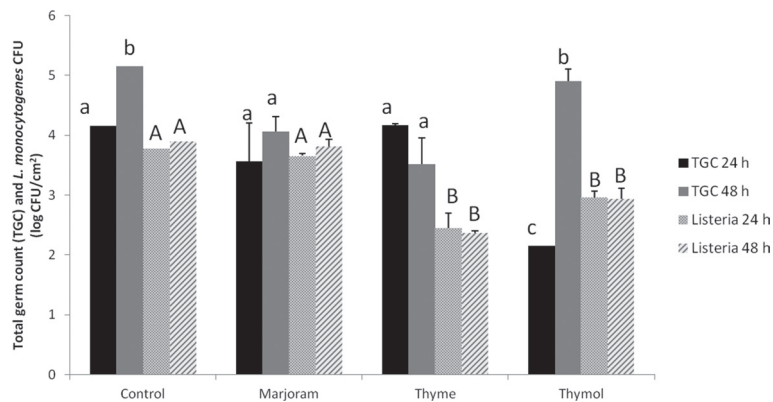


Fig. 2. Total germ counts (TGC) and *Listeria* CFU after marinating of the samples washed with water. Lower cases show comparison between EOs and the control sample in case of TGC and upper cases, comparison between *L. monocytogenes* counts

treated samples TGC remained at the level of the 24 h values. Compared to the control (4.8 log CFU/cm²) *Listeria* counts were reduced with 1.6 log after marinating with marjoram and 2 log after thymol. Thyme EO completely reduced CFU (Fig. 1).

For washed samples thymol was the best inhibitor reducing TGC with 2 logs after 24 h. *Listeria* CFU was reduced with approximately 1 log ($P < 0.001$) after marinating with thyme and thymol (thyme was more effective). After 48 h TGC was reduced by marjoram and thyme ($P < 0.05$). Thymol was not efficient in this case but it exhibited a good anti-listerial effect along with thyme ($P < 0.001$) (Fig. 2).

Microbiological changes in the marinating solutions of inoculated chicken breast fillets

Results of the marinating solutions are presented in Figures 3 and 4. TGC values of the unwashed samples stored for 24 h were reduced by all marinating agents. A 0.6, 2.5 and 3 log reduction was obtained after marjoram ($P < 0.05$), thyme ($P = 0.003$) and thymol ($P < 0.001$) treatments, respectively. Thymol reduced *Listeria* CFU with 2 logs while the other two EOs completely inhibited growth. Marjoram was not effective against TGC in the 48-h samples ($P > 0.05$) but thyme and thymol reduced cell counts significantly (1.7 and 2 log reductions). No listerial growth could be detected in solutions with marjoram and thyme EOs while thymol resulted in a 2 log reduction of cell count (Fig. 3).

Washed samples stored for 24 h showed 1, 6 and 5 logs reduction after marinating with marjoram, thyme and thymol, respectively. *Listeria* could not be detected in either of the solutions. Only thymol was effective in reducing TGC values after 48 h

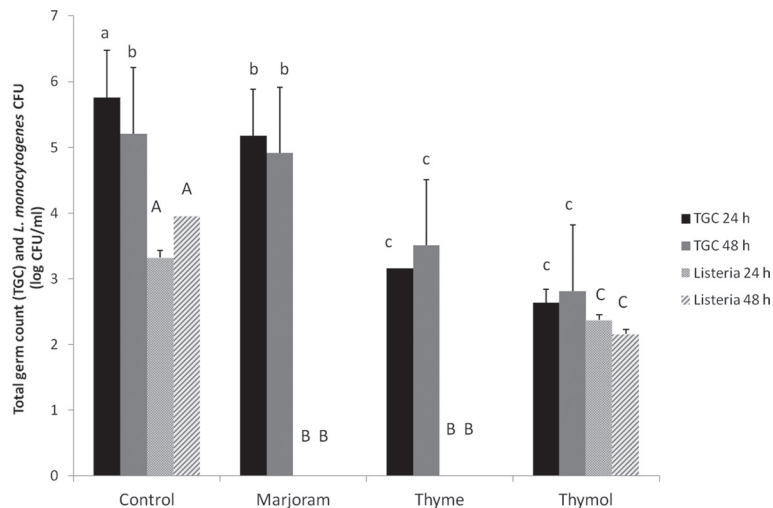


Fig. 3. Total germ counts (TGC) and *Listeria* CFU after marinating in the marinating solutions of the unwashed samples. Lower cases show comparison between EOs and the control sample in case of TGC and upper cases, comparison between *L. monocytogenes* counts

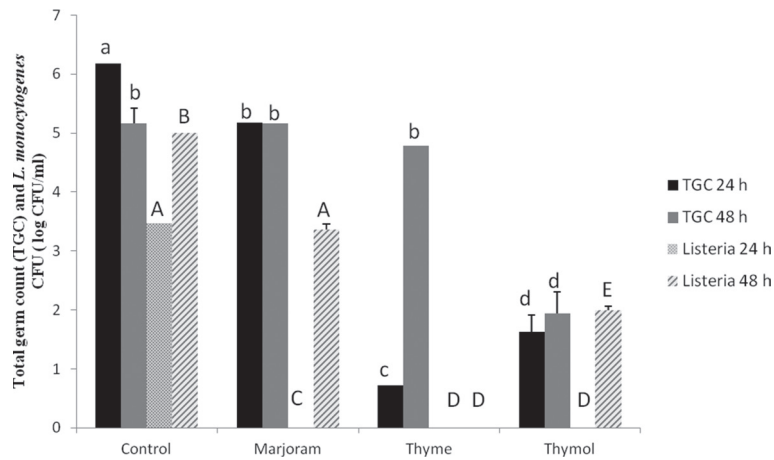


Fig. 4. Total germ counts (TGC) and *Listeria* CFU after marinating in the marinating solutions of the washed samples. Lower cases show comparison between EOs and the control sample in case of TGC and upper cases, comparison between *L. monocytogenes* counts

storage (3 logs) and *Listeria* was reduced in all marinating solutions; thyme being the most efficient EO (Fig. 4).

Sensory evaluation of the marinated chicken breast fillets

In case of marjoram, sensory panelists rated the fillets with an average score of 3.4 for odour and 2 for taste. These results differed significantly from the control sample in both their odour ($P = 0.01$) and taste ($P < 0.001$). It was also stated that the meat

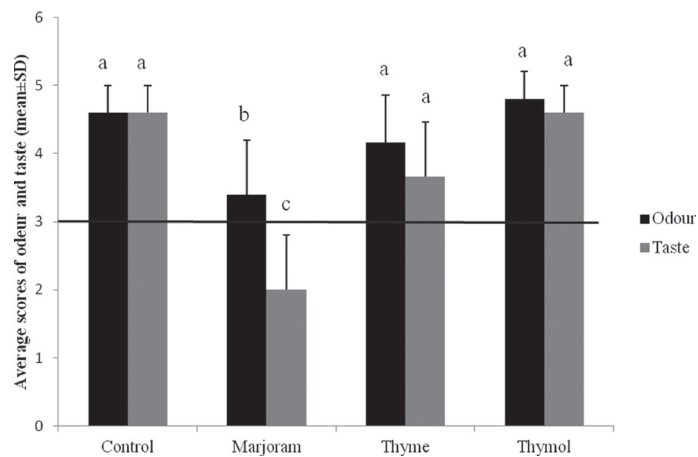


Fig. 5. Average scores of the sensory evaluation of chicken fillets after marination. Values are mean±SD and significant differences are marked with low cases. The limit of acceptability is marked by a black line

had an unpleasant aftertaste. Thyme and thymol had similar acceptability to the control sample regarding odour and taste scoring between 3.6 and 4.8 for both parameters. In these cases panelists stated that the meat had a nice spicy taste which harmonized with the meat. For some of the panelists the meat samples were too salty (Fig. 5).

DISCUSSION

Food contamination is a serious public health problem that may be better controlled with the use of natural preservatives. EOs and thymol used in these experiments were chosen because these flavours are frequently used for seasoning the chicken meat. Marinating with MIC/2 values of marjoram EO gave an unpleasant aftertaste, furthermore, it had no significant effect in reducing neither TGC nor *Listeria* CFU. On the other hand, we could determine a dose of thyme EO which combines antimicrobial efficiency with a pleasant flavour (1 mg/mL for thyme and 0.2 mg/mL for thymol). This shows that the use of EOs is limited and it is difficult (but not impossible) to find a concentration where both antimicrobial effect and pleasant flavour is combined.

Washing of the meat had apparently no effect on TGC counts. On the other hand listerial growth during the 24 h pre-incubation increased after washing. Explanation of this finding would require further studies. Though it was found earlier that psychrotrophic lactobacilli on the meat surface could have inhibitory activity against *L. monocytogenes* [5, 17], however, bacteria isolated from the meat surface in our study did not show any antagonism to *Listeria*.

Results show that in the marinating solutions TGC values were higher than on the surface of the meat. It is important to note that thyme and thymol inhibited growth in the marinade more effectively than on the meat surface. This result is in agreement with literature data stating that planktonic cells are more susceptible to inhibitors [10]. Washing poultry meat before use is discouraged by the authorities to avoid spreading of pathogens. Our study also proved that washing was not efficient in lowering TGC and pathogenic germ counts so there is no reason to wash the meat before further use.

The use of EOs as spices and preservatives at an industrial level requires detailed knowledge of their not-standardized chemical composition. Changes in their components occur because of environmental factors like temperature, humidity, harvest time, effect of soil properties, the maturation of the plant [21, 22]. It is highly recommended to measure chemical compositions, antimicrobial effect and the interaction of EOs with the food matrix before adding them to food products.

According to our findings, thyme EO and thymol could keep *L. monocytogenes* and TGC counts low on marinated raw chicken fillets at proper refrigerated conditions without having negative effect on organoleptic properties.

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