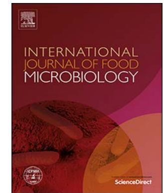
International Journal of Food Microbiology 258 (2017) 58–67

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro



Inhibition of *Salmonella* by thyme essential oil and its effect on microbiological and sensory properties of minced pork meat packaged under vacuum and modified atmosphere

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A R T I C L E I N F O

Keywords:
Thymus vulgaris
Pork
Packaging
Salmonella
Lab
Headspace

A B S T R A C T

The antibacterial activity of thyme essential oil (TEO) was evaluated against four serovars of *Salmonella* (*S.* Enteritidis, *S.* Typhimurium, *S.* Montevideo and *S.* Infantis), experimentally inoculated (10^6 CFU/g) in minced pork, which was treated with different concentrations of the TEO (0.3%, 0.6% and 0.9%) packaged under vacuum or MAP (30%O_2/50%CO_2/20% N_2) and stored at 3 ± 1 °C for 15 days. GC–MS analysis of the TEO was performed in order to determine composition, and the predominant constituent was thymol (50.48%), followed by *p*-cymene and linalool. The minimum inhibitory concentration was determined for each *Salmonella* serovar studied. Among the tested active compounds, thymol and carvacrol exhibited the greatest inhibitory effect followed by TEO, with minimum inhibitory concentrations of 320 to 640 μg/ml. *S.* Enteritidis was the most sensitive serovar. During the storage period, *Salmonella* counts in pork were reduced by 1.69–4.05 log CFU/g. The influence of TEO on *Enterobacteriaceae*, lactic acid bacteria and total viable count was determined in control mince with no added *Salmonella*. The most pronounced antibacterial effect was achieved by the combination MAP and 0.9% TEO. Although the antibacterial activities of all studied concentrations of TEO in pork were evident and significant ($P < 0.05$), sensory analysis showed that 0.3% TEO was the most acceptable to trained panellists.

1. Introduction

Despite a slight decrease in production in some regions over past years, globally, pork meat is still the most widely consumed meat (FAO, 2014). As other red meats, pork meat is a rich source of high value proteins, fats, vitamins and microelements. Therefore, it plays an important role in human diets but is also an ideal substrate supporting the growth of spoilage and pathogenic bacteria including *Salmonella* spp. (Mataragas et al., 2008). In Europe, pork meat and derived products are recognized as the main sources of *Salmonella* infection in humans, followed by eggs, broilers, and turkeys (Delibato et al., 2014; EFSA, 2013). Contamination of pig carcasses can occur during slaughter by the contents of the intestine or lymph nodes, but *Salmonella* can also enter the meat as a result of cross-contamination and recontamination events during any step of meat production, processing, transport, storage and even in domestic environments during handling and preparation of food (Castelijn et al., 2013). Along with other foodborne pathogens, *Salmonella* is the cause of millions of episodes of illness annually worldwide, presenting not only a health but also an economic problem in both developed and developing countries (EFSA, 2010). *Salmonella* Enteritidis and *Salmonella* Typhimurium are the most frequently reported serotypes in the incidence of human salmonellosis in both EU and United States (Carrasco et al., 2012), but the incidence of *Salmonella* Infantis and other serotypes is increasing.

Pork meat is not only a common source of pathogenic microorganisms but is also one of the most susceptible meats to deterioration because it contains more bacteria than other meats (Mataragas et al., 2008). In recent years, consumers' concern and increasing demand for healthier and natural food resulted in interest in alternative methods to prevent foodborne diseases and retard deterioration. Essential oils (EOs) have been used since ancient times, but in past few decades have been investigated as food additives. Among some other EOs, thyme EO (TEO) is categorized as GRAS (Generally Recognized as Safe) by the Food and Drug Administration (Manso et al., 2014). TEO is one of the EOs most commonly used for food preservative purposes (Nabavi et al., 2015). Due to its high phenolic compound content, TEO is a potent

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http://dx.doi.org/10.1016/j.ijfoodmicro.2017.07.011
Received 17 February 2017; Received in revised form 11 June 2017; Accepted 18 July 2017
Available online 20 July 2017
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antibacterial. TEO showed an antibacterial effect against a large range of microorganisms in vitro, but only a few studies evaluated its antimicrobial activity in meat models. Furthermore, there are no literature data about the effect of TEO against less common *Salmonella* serovars like *S*. Infantis and *S*. Montevideo.

Therefore the aim of this study was to evaluate the efficacy of TEO on *Salmonella*, *Enterobacteriaceae*, lactic acid bacteria and the total viable count (TVC) in minced pork meat packed under vacuum and modified atmosphere and stored at 3 ± 1 °C.

2. Materials and methods

2.1. Thyme essential oil

The TEO, extracted by the steam distillation method, was purchased from the manufacturer Herba doo (Belgrade, Serbia). TEO was kept in dark glass bottles at 4 °C.

2.2. Determination of TEO composition

TEO was analysed by gas chromatography with electron-ionization mass-selective detector (Agilent Technologies 6890 N + 5975B). Amounts (1 μl) of TEO were injected into a split/splitless inlet at 250 °C, with a split ratio 1:10. Helium (purity 99.999%) was used as a carrier, with a constant flow of 1 ml/min. The separation was achieved on a 30mx0.25mmx0.25 m semipolar HP-5 ms capillary column (Agilent Technologies) made of polydimethylsiloxane with 5% of phenyl groups, using the following temperature program: start at 50 °C, 8 °C/min to 120 °C, 15 °C/min to 230 °C, 20 °C/min to 270 °C and hold for 16.9 min (total run time 35 min). Elute was delivered to the mass spectrometer (Agilent Technologies series 5975) via a transfer line held at 280 °C. Ion source temperature was 230 °C, electron energy 70 eV, and quadrupole temperature 150 °C. Data were acquired in Scan mode (m/z range 35–400). Data were analysed by Agilent MSD ChemStation software and AMDIS (Automated Mass Spectral Deconvolution and Identification System) in conjunction with NIST MS Search software. The compounds were identified by mass spectra comparison with libraries (Wiley Registry of Mass Spectral Data 7th ed. (McLafferty, 2005), and NIST/EPA/NIH Mass Spectral Library 05 (NIST/EPA/NIH, 2005)) and confirmed by comparison of linear retention indices (LRI) with literature data (Adams, 2001). Diesel oil was used as a standard for determination of retention indices. Relative amounts of components, expressed in percentages, were calculated by normalization measurement according to peak areas in total ion chromatograms.

2.3. Salmonella serovars and inoculum preparation

Four serovars of *Salmonella enterica* (*Salmonella* Enteritidis ATCC13076, *Salmonella* Typhimurium ATCC 14028, *Salmonella* Montevideo 1, 3, 19: g, s, t and *Salmonella* Infantis 6, 7: r: 1, 5) were used in this study. These serovars were stored in Brain Heart Infusion (BHI; Merck, Germany) with 20% glycerol at − 80 °C until needed. One ml of each frozen *Salmonella* serovar was added to 10 ml of Brain Heart Infusion (BHI, Merck, Germany) and incubated at 37 °C for 24 h. The *Salmonella* serovars were then streaked on Xylose Lysine Tergitol-4 Agar (XLT_4) (Oxoid, UK) to verify their characteristics. In order to obtain a second subculture, isolated black colonies were picked from the XLT_4 plates and inoculated into BHI tubes (1 colony per tube) and further incubated for another 24 h at 37 °C. After incubation, the cultures were centrifuged at 5000 × g (Eppendorf, Hamburg, Germany) for 10 min, and suitable dilutions were prepared in BHI. Cocktails of *Salmonella* serovars were prepared by combining equal portions of standardized cell suspensions to yield approximately 6 log CFU/ml of each serovar in the mixture. *Salmonella* counts were determined by serial dilution and subsequent enumeration on XLT_4.

2.4. Determination of minimum inhibitory concentration

Susceptibility of the *Salmonella* serovars to active compounds was investigated by the broth microdilution method (CLSI, 1999, 2009). The broth microdilution method was performed in sterile U-bottom microtitre plates (Spektar, Serbia). The inoculum density was set to 0.5 McFarland (approximately 1-2 × 10^8 CFU/ml), diluted 10 times (1–2 × 10^7 CFU/mL) in sterile saline and 5 μl of this suspension was inoculated in 0.1 ml of Cation Adjusted Mueller-Hinton Broth (CAMHB; Becton, Dickinson and Company, Sparks, USA) to reach the final inoculum of 5 × 10^4 CFU/well. Active compounds (thymol, carvacrol, cinnamaldehyde, eugenol - Essentico, Kula, Serbia; *p*-cymene - Sigma-Aldrich, USA) were diluted in DMSO (Serva, Heidelberg, Germany) and added to CAMHB at levels from 2560 μg/ml to 1.25 μg/ml by two-fold dilution in 96-well microtitre plates. After inoculation, plates were incubated at 37 °C for 24 h. Minimal inhibitory concentration (MIC) was determined as the lowest concentration of an active compound that prevented visible growth of a *Salmonella* serovar in the broth dilution susceptibility test (CLSI, 2006). For control, amikacin (Sigma-Aldrich, USA) was used in the range of 64 to 0.03 μg/ml.

2.5. Sample preparation, packaging and storage condition

Pork muscles from the legs of different carcasses of Yorkshire × Landrace crossbreeds were obtained 48 h post-slaughter from a local slaughter house. Connective tissues and visible fat were trimmed, after which the pieces of meat were minced aseptically in a grinder with 4 mm perforations in the grinding plates. The meat was transported to the laboratory under refrigerated conditions within an hour.

A *Salmonella* cocktail containing 6 log CFU/g (described above) was used to inoculate half of the mince. Both experimentally contaminated and non-contaminated halves of the minced meat were divided into four equal parts. TEO at different concentrations (0.3%, 0.6% and 0.9%, respectively) was added to the first, second and third part of both halves, while the forth parts remained without TEO. Then, all mince portions were divided in two more parts, and packaged either in vacuum or with a gas mixture containing 50%CO_2/30%O_2/20%N_2, with a 2:1 gas volume to meat ratio. A high speed thermoforming packaging machine, Multivac R 245 (Multivac Verpackungsmaschinen, Wolfertschwenden, Germany), was used for MAP packaging, where the air was replaced by a gas mixture containing 50%CO_2/30%O_2/20%N_2. For vacuum packaging, a Multivac C 500 machine (Multivac Verpackungsmaschinen, Wolfertschwenden, Germany) was used. Mince samples were packed in a PA/EVOH/PE foil (polyamide/ethylene vinyl alcohol/polyethylene Dynopack, POLIMOON, Kristiansand, Norway), with low permeability to gas. The degree of permeability to O_2 was 3.2 cm^3/m^2/day at 23 °C, to N_2 was 1 cm^3/m^2/day at 23 °C, to CO_2 was 14 cm^3/m^2/day at 23 °C and to water vapour was 15 g/m^2/day at 38 °C. Prepared groups are presented in Table 1. All samples of minced meat weighing 100 ± 5 g were refrigerated at 3 ± 1 °C and examined on day 0 and on days 3, 6, 9, 12 and 15 of storage.

2.6. Microbiological analyses

Minced meat was analysed for *Salmonella* spp. on day 0 in order to determine the presence or absence of this pathogen, while experimentally contaminated mince samples were analysed for *Salmonella* spp. on day 0 and on days 3, 6, 9, 12 and 15 of storage. Mince which had not been inoculated with *Salmonella* was analysed for total viable count (TVC - mesophiles, 30 °C), total *Enterobacteriaceae* count and lactic acid bacteria (LAB) count on day 0 and on days 3, 6, 9, 12 and 15 of storage. For bacterial enumeration, 20 g of mince was weighed out aseptically after pack opening, transferred into sterile Stomacher bags and 180 ml of Buffered Peptone Water (BPW) (Merck, Germany) was added to each sample. Mince samples were homogenized in a Stomacher blender

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