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Original scientific paper

Inhibitory effect of thyme and oregano essential oils and some essential oil components on *Salmonella* Senftenberg *and Salmonella* Give

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A b s t r a c t: Salmonella is a pathogen of public concern causing health and economic problems worldwide. Salmonella Enteritidis and Salmonella Typhimurium are the serotypes most commonly recognized as causes of human salmonellosis, which is why research is mainly dedicated to prevention or inhibition of these frequently reported serotypes, while less attention is dedicated to the uncommon Salmonella serotypes. Outbreaks of salmonellosis caused by rarer subspecies of Salmonella are increasing, which is why their control is needed. Essential oils derived from plants have gained attention mainly due to their antibacterial properties and potential to be used as a replacement for synthetic additives in the food industry. To the best of our knowledge, there are no literature data about the effect of essential oils on Salmonella Give. Therefore, the aim of this study was to evaluate the effect of thyme and oregano essential oils and thymol, carvacrol, cinnamaldehyde and eugenol on Salmonella Senftenberg and Salmonella Give. Results showed that there were no differences between the susceptibility of the examined Salmonella serovars to these essential oils and active compounds. Oregano essential oil, thymol and carvacrol exhibited greater antibacterial activity, followed by cinnamaldehyde, while the Salmonella serovars examined were most resistant to the effect of eugenol.
Keywords: *Salmonella, thymol, carvacrol, cinnamaldehyde, eugenol.*

Introduction

Foodborne diseases are an important cause of morbidity and mortality worldwide (*Van et al.,* 2007). Bacterial pathogens are considered to be the most common agents causing foodborne diseases and among foodborne bacteria, *Salmonella* is the most common cause of illness after *Campylobacter* (*Carrasco et al.,* 2012; *de Silva et al*., 2013). It is estimated that non-typhoidal salmonellosis is the cause of 155,000 deaths annually and of 93.8 million reported cases, 80.3 million are foodborne (*Majowicz et al*., 2010). The major vehicles of this pathogen are eggs, poultry and pork, as well as other types of meat and meat products, but *Salmonella* is often found in low-moisture foods (powdered milk, chocolate, peanut butter, infant formula), vegetables, spices, seafood, milk and milk products (*Carrasco et al*., 2012; *Pires et al*., 2014). *Salmonella* Enteritidis and *Salmonella* Typhimurium followed by *Salmonella* Infantis are the most frequently reported serotypes in human salmonellosis, but other serotypes were also involved in salmonellosis outbreaks (*de Freitas Neto et al*., 2010; *Carrasco et al.*, 2012). The incidence of *Salmonella* infections with more rare serovars is increasing (*David et al*., 2007). *Salmonella* Senftenberg is not one of the serotypes most commonly associated with human infection but is a pathogen of public interest due to its high heat resistance (*Pezzoli et al*., 2008; *Gurman et al*., 2016). *Salmonella* Give was identified as the cause of some cases of illness and minced pork and infant milk formula were detected as sources of infection (*Girardin at al*., 2006; *Berger*, 2015). This serotype was found on beef carcasses, refrigerated and pulp meat (*David et al*., 2007; *Perez-Montaño et al*., 2012). Although infection with this serotype is rare, the hospitalisation rates for patients infected with *S*. Give are higher compared to those infected with *S*. Enteritidis. This possibly indicates that this serotype has a higher virulence compared to other non-typhoidal *Salmonella* spp. (*Girardin et al*., 2006).

Salmonellosis is usually a self-limiting disease and symptoms include fever, chills, nausea, vomiting, abdominal cramping, and diarrhoea (*Chen et*

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al., 2013). Infants, young children, the elderly and the immunocompromised are at particular risk for bacteraemia which occurs in 5–10% of infected persons, and may progress to focal infection including meningitis, bone and joint infection (*Chen et al*., 2013; *Crump et al.,* 2015).

Therefore, *Salmonella* infections are a major human public health and economic problem in both developed and developing countries (*EFSA*, 2010) and novel strategies and methods for control of this pathogen are needed.

A number of studies have reported essential oils to be effective antimicrobials with potential application in meat and in the general food industry to increase the safety of these products. Although *Salmonella* is one of the most investigated pathogens, there are little or no data about the effects of essential oils or their components on uncommon *Salmonella* serotypes. Considering the above, the aim of this study was to evaluate the effects of thyme and oregano essential oils, thymol, carvacrol, eugenol and cinnamaldehyde on *Salmonella* Senftenberg and *Salmonella* Give.

Materials and Methods

Essential Oils and Active Compounds

Thymol, carvacrol, cinnamaldehyde and eugenol were purchased from the manufacturer (Essentico, Kula, Serbia). Oregano and thyme essential oils, extracted by the steam distillation method, were purchased from the manufacturer (Herba doo, Belgrade, Serbia). The major components of thyme (*Thymus vulgaris*) essential oil, determined by GC-MS analysis, were thymol and p-cymene followed by linalool, γ-terpinene and 1,8-clineole and of oregano (*Origanum vulgare*) essential oil were carvacrol followed by *p*-cymene, trans-β-caryophyllene, linalool, γ-terpinene and thymol. Other chemical compounds were in lower concentrations. Essential oils were kept in dark glass bottles at 4°C.

Antibacterial Assay

The antibacterial effects of oregano and thyme essential oils, thymol, carvacrol, eugenol and cinnamaldehyde on *Salmonella enterica subsp. enterica* serovar Senftenberg (6.7:g,m,s :-) and *Salmonella enterica subsp. enteric* serovar Give (3,10:l.v:1.7) were studied. *Salmonella* Senftenberg (Veterinary Institute Subotica, Serbia) was isolated from animal feed and *Salmonella* Give (Veterinary Institute Subotica, Serbia) from poultry meat.

The susceptibility of *Salmonella* isolates to essential oils and active compounds was investigated by the broth microdilution method (*CLSI* 1999; *CLSI* 2009). Broth microdilution method was performed in sterile U-bottom microtitre plates (Spektar, Serbia). The inoculum density was set to 0.5 McFarland (approximately $1–2 \times 10^8$ cfu mL^{-1}), diluted 10 times ($1–2 \times 110^7$ cfu mL^{-1}) in sterile saline and 5 µL of this suspension was inoculated in 0.1 mL of CAMHB-Cation Adjusted Mueller-Hinton Broth (Becton, Dickinson and Company, Sparks, USA) to reach a final inoculum of 5 x 10^4 cfu $well^{-1}$. Active substances were diluted in DMSO (Serva, Heidelberg, Germany) and added to CAMHB at levels from 2560 µg mL^{-1} to 1.25 µg mL^{-1} by two-fold dilution in 96-well microtitre plates. After inoculation, plates were incubated at 37°C for 24 hours. Minimal inhibitory concentration (MIC) was determined as the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in broth dilution susceptibility test (*CLSI*, 2006). From wells without visible growth, 10 µL was subcultivated onto CAMH Agar and incubated at 37°C for 24 hours. Growth of less than five colonies was taken as the minimal bactericidal concentration (MBC) as it represented a kill ratio of over 99.9% (CLSI, 1999). Amikacin (Sigma-Aldrich, USA) in the range of 64–0.03 µg mL^{-1} was used as control.

Results and Discussion

Results of the antimicrobial activity of thyme and oregano essential oils, thymol, carvacrol, cinnamaldehyde and eugenol on the *Salmonella* serovars studied are presented in Table 1.

The antibacterial effects of the essential oils used in this study were previously reported (*Boskovic et al.*, 2015). As hydrophobic liquids, essential oils interact with the lipid membrane of bacterial cells, causing the collapse of the proton motive force and depletion of the ATP pool, thus changing the membrane permeability and leading to leakage of the inner cell components and eventually to cell death (*Ultee et al.*, 2002; *Burt*, 2004; *Bajpai et al.*, 2012). Essential oils also affect potassium ion reflux and cause coagulation of cytoplasm (*Burt*, 2004; *Bakkali et al*, 2008).

Results from a number of studies confirmed that Gram-negative bacteria, including *Salmonella* spp. are more resistant to effects of essential oils than Gram-positive bacteria due to their outer membrane which covers the cytoplasmic membrane and their peptide-glycan layer, which acts as a barrier against

Table 1. The minimum inhibitory concentrations of oregano and thyme essential oils and active compounds against *Salmonella* spp.

	Minimum inhibitory concentration (µg mL^{-1})						
	Essential oils		**Active compounds**				**Antibiotic**
	Oregano	Thyme	Thymol	Carvacrol	Cinnamaldehyde	Eugenol	Amikacin
S. Senftenberg	320	640	320	320	640	1280	1
S. Give	320	640	320	320	640	1280	0.25

hydrophobic macromolecules (*Holley and Patel*, 2005; *Hyldgaard et al*., 2012; *Esteban et al*., 2013).

In the current study, results showed that both *Salmonella* serovars were equally sensitive to oregano and thyme essential oil, thymol, carvacrol, cinnamaldehyde and eugenol, showing minimum inhibitory concentrations of 320 µg mL^{-1}, 640 µg mL^{-1}, 320 µg mL^{-1}, 320 µg mL^{-1}, 640 µg mL^{-1} and 1280 µg mL^{-1}, respectively.

The high antimicrobial activity of thyme and oregano essential oils has been attributed to their phenolic components such as thymol and carvacrol (*Bajpai et al.*, 2012; *Bassolé and Juliani*, 2012). In the present study, oregano essential oil exhibited a stronger antibacterial effect than thyme essential oil, probably as a result of the higher content of phenolic compounds (data not shown). Because essential oils are complex mixtures containing a number of components, the antimicrobial activity cannot be attributed to single compound (*Bajpai et al*., 2012; *Boskovic et al*., 2013). Nevertheless, in the present study, carvacrol and oregano essential oil (which comprised 77.6% carvacrol) exhibited the same antibacterial effect. Other authors also reported the antibacterial effects of thyme and oregano essential oil on *Salmonella* spp. (*Bajpai et al.*, 2012). Still, most of these studies have been conducted on *S*. Typhimurium and *S*. Enteritidis and so there are few literature data about the effect of essential oils on *S*. Senftenberg. *Cherrat et al*. (2014a) reported the antibacterial effect of *Laurusnobilis* and *Myrtuscommunis* essential oils against *S*. Senftenberg. In their study, *Laurusnobilis* showed greater antimicrobial activity but the reported MIC values were much higher than those found for essential oils in the present study. *Menthapulegium*, *Saturejacalamintha* and *Lavandulastoechas* also exhibited an antimicrobial effect on *S*. Senftenberg but in higher concentrations, 4 µL mL^{-1}, 14 µL mL^{-1} and 14 µL mL^{-1}, respectively (*Cherrat et al.,* 2014b). *Nanasombat and Lohasupthawee* (2005) examined the effect of different essential oils obtained from spices on nine serotypes of *Salmonella* which were potential pathogens and most commonly isolated from fresh and fermented meat, including *S*. Senftenberg. This serotype was the most or equally sensitive to cardamom, coriander, cumin, kaffir lime peel and ginger essential oils (MIC 0.2 µL mL^{-1}) less sensitive to mace and nutmeg essential oils (MIC 8.3 µL mL^{-1}) and most resistant to garlic (MIC 47.6 µL mL^{-1}), kaffir lime leaf and holy basil essential oils (MIC >62.5 µL mL^{-1}). The MICs obtained for essential oils in their study were higher than those determined in the present study but it should be noted that they used an inoculum concentration of 10^7cfu mL^{-1} in contrast to our study where an inoculum concentration of 10^4 cfu well^{-1} was used. Differences between the effects of essential oils towards bacteria are mainly attributed to its chemical profile (*Burt*, 2004; *Boskovic*, 2013).

Strain biodiversity, among other factors, influences the antimicrobial resistance of *Salmonella* (*Mazzarrino et al*., 2015). Differences were not observed between tested serovars of *Salmonella* in the present study, but as *Boskovic et al*. (2015) reported, the same essential oils were shown to be more effective against *S. Typhimurium* and thyme essential oil was more effective against *S*. Enteritidis. Lu and Wu (2010) did not find differences between the susceptibility of four *Salmonella* serovars (*S. Kentucky, S. Senftenberg, S. Enteritidis* and *S. Typhimurium*) to thyme essential oil, thymol and carvacrol. They obtained higher MIC values for thyme essential oil than those in the present study, and thymol exhibited the strongest antibacterial activity against all four *Salmonella*. Results from the present study showed that thymol and carvacrol exhibited stronger antibacterial activity than cinnamaldehyde and eugenol, with an obtained MIC value of 320 µg mL^{-1} for both *Salmonella* serovars.

A number of studies showed that among constituents of essential oils, carvacrol and thymol exhibited the greatest antibacterial activity which is why these substances are the most investigated and mechanism of their action is well described (*Burt*, 2004). Thymol is structurally analogous to carvacrol, but the locations of the hydroxyl groups are at a different location on the phenolic ring. Both phenols interact with the outer membrane of gram-negative bacteria, releasing lipopolysaccharides and increasing the permeability of the cytoplasmic membrane to ATP and potassium ions (*Burt*, 2004; *El Abed et al*., 2014). As mentioned above, a high percentage of phenolic compounds, including eugenol as well as carvacrol and thymol, are considered to be responsible for the antimicrobial activity of essential oils (*Burt*, 2004), but in the present study, eugenol exhibited the lowest antimicrobial activity against both tested *Salmonella* serovars with an obtained MIC value of 1280 μg mL^{-1}. Apart from essential oils containing phenols, essential oils containing significant amounts of aldehydes, such as cinnamaldehyde, showed high antibacterial activity (*Bassolé and Juliani*, 2012). The mode of action of cinnamaldehyde, the main component of cinnamon essential oil, is not still fully understood. Cinnamaldehyde, depending on the added concentration, inhibits different enzymes involved in cytokinesis or less important cell functions, acts as an ATPase inhibitor and perturbs cell membranes (*Hyldgaard et al*., 2012; *Shen et al*., 2015). It has been suggested that cinnamaldehyde inhibits cytokinesis (*Hyldgaard et al*., 2012). In the present study, cinnamaldehyde exhibited a moderate antibacterial effect. In contrast, *Zhou et al*. (2007) reported that cinnamaldehyde (MIC 200 mg L^{-1}) was more effective against *S*. Typhimurium than thymol and carvacrol (400 mg L^{-1}). The MICs reported in the present study for thymol and carvacrol were lower and these results may be caused by different sensitivity between the *Salmonella* serovars studied. Amikacin was used as a positive control. As was expected, amikacin showed a greater antimicrobial effect than the essential oils and active compounds. *S*. Give was more susceptible to amikacin than *S*. Senftenberg. Amikacin has a different mechanism of action compared to essential oils; while essential oils act mainly on the cell membrane, amikacin inhibits translation and amino acid misincorporation, and thus bacterial protein synthesis, by binding to rRNA (*López-Díez et al.*, 2005). Different modes of action could explain differences between susceptibility of *Salmonella* serovars to the antimicrobial substances examined.

Conclusion

Despite invested efforts to control *Salmonella*, this pathogen is still a public health problem. As many pathogens, *Salmonella* can also be inhibited by essential oils. The results of this study showed that oregano and thyme essential oils and active compounds, in differing concentrations, exhibited antibacterial effects on *Salmonella* Senftenberg and *Salmonella* Give. Oregano essential oil, thymol and carvacrol exhibited the greatest effect on both tested *Salmonella* serovars. Taking into account that there are little or no data about the effect of essential oils on the two examined serovars of *Salmonella*, further research should be undertaken in food substrates to confirm the antimicrobial effects of essential oils and active compounds on these *Salmonella* serovars.

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