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Effect of *Thymus vulgaris* on Initial Cell Attachment and Preformed Biofilm of *Salmonella* Enteritidis

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Bacteria within a biofilm have a distinct phenotype from planktonic cells and generally show higher tolerance to antimicrobial agents, such as disinfectant treatments and antibiotics. Due to the increased tolerance against antimicrobial treatments, biofilms are hard to eradicate and they cause all kinds of problems in medical and industrial settings. Therefore, the purpose of this study was to examine the influence of *Thymus vulgaris* essential oil on the initial adhesion and preformed biofilm of *Salmonella* Enteritidis. Results for antimicrobial activity of *Th. vulgaris* essential oil obtained using broth microdilution method against *S.* Enteritidis were identical for all tested isolates (MIC/MFC=0.156/0.3125 μL/mL). In order to understand the anti-biofilm action of essential oil (EO), its effect was tested on both the initial cell attachment by planktonic cells as well as on preformed biofilms. Obtained results indicated that the effect of *Th. vulgaris* essential oil on initial cell attachment of tested isolates SE4, SE5, SE7 and SE9 was dosage dependent manner, although even at 0.5×MIC biomass attachment was reduced by 74.2%, 77.7%, 73.6% and 70.1%, respectively. Fairly high, but not complete, inhibition of cell attachment was achieved using 1×MIC of this EO. Using 1×MIC, biomass attachment of tested isolates SE4, SE5, SE7 and SE9 was reduced for 91.4%, 90.2%, 90.6% and 88.4%, respectively. When the same EO was tested against a preformed biofilm, its inhibitory effect was reduced greatly.

Key words: *Salmonella* Enteritidis, biofilm, *Thymus*.

The ability of biofilm formation is an ancient property of bacteria and other prokaryotes that represents, from the evolution point of view, the survival strategy in variable and often highly unfavorable conditions of the environment. Contrary to characteristics that bacteria show during their growth in the media abounding with nutritive substances, bacteria in biofilms show different properties in terms of genes expression and growth characteristics. Due to the presence of these differences, bacteria in biofilms show an increased resistance to antibiotics and disinfectants and that is why it is almost impossible today to treat infections caused by biofilms with conventional antibiotics, just as it is not possible to control bacteria in the hospital environment and places of food production using disinfectants in recommended concentrations. In medical and industrial microbiology, the concept of biofilms formed by bacteria was accepted during the 1990s

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and it is currently one of the greatest challenges in the field of protection of human health and production of healthy safe food.

A large number of studies confirmed that biofilms formed by bacteria on different surfaces in food industry plants make a long-term source of contamination of foodstuffs, not only with bacteria causing their spoilage but also with food-borne pathogen species such as *Salmonella* spp., *Campylobacter* spp., *Escherichia coli* and *Listeria monocytogenes*. It is known that some food-borne pathogens in food production plants may exist throughout several months, even years. These strains are known as "house strains", and the assumption is that the existence of such strains is enabled due to their ability to form biofilms [1]. Several studies confirmed the ability of adherence and biofilm formation of food-borne pathogens on different types of materials that are usually used in food industry [2-5]. Biofilms of food-borne pathogens are found on conveyer belts, cutting and packing machines and other surfaces that get in contact with food. Having in mind the consequential effect that biofilm formation may have in food industry, the biofilm control in food industry plants becomes the imperative in production of healthy safe food.

The efficiency of disinfectants and recommended concentration are the results of tests conducted on broth bacteria cultures. While their application on broth cultures is efficient up to 100% [6, 7] the application of the same concentrations on bacteria in biofilms shows inefficiency so that their elimination requires much larger concentrations. Because of these properties of biofilm-associated cells there is a large and increasing interest in substances, which inhibit specific processes in the initial phase of biofilm formation and therefore prevent the formation of mature biofilms with its elevated resistance against biocides and disinfectants [8]. A large number of research are based on finding the potential biological solutions for biofilm prevention and elimination that include antimicrobial compounds of plant origin [9], enzymes, phage, inter-species competitors, or antimicrobial compounds produced by microorganisms [10]. This study was aimed to evaluate the influence of *Th. vulgaris* essential oil on initial cell attachment and preformed biofilm of *Salmonella* Enteritidis.

MATERIALS AND METHODS

Plant material

Plant material *Thymus vulgaris* used in this study was obtained from Institute of Medicinal Plant Research Dr. Pancic, Belgrade, Serbia and voucher specimen was deposited in the herbarium of the Institute of Medicinal Plant Research Dr. Pancic.

Isolation of the essential oil

The essential oil (EO) was isolated from dried plant material (100g) by hydro-distillation according to the standard procedure. Distillation was performed using Clevenger type apparatus, for 4 hours. The resulting EO was dried over anhydrous sodium sulfate and stored in sealed dark vials at 4°C. The yield of EO was expressed in volume percent (% v/w) that was calculated relative to 100 g of dried plant material.

Microorganisms

A total of four *S. enterica* strains of serovar Enteritidis (SE4, SE5, SE7 and SE9) were used in this study. Strains were isolated from fecal samples obtained from the Veterinary Institute in Kraljevo. Serological typing and verification of *Salmonella* isolates was carried out in the National Reference Laboratory for *Salmonella*, *Shigella*, *Vibrio cholera* and *Yersinia enterocolitica*, Institute of Public Health of Serbia "Dr Milan Jovanovic Batut", Belgrade, Serbia.

Preparation of bacterial suspension

S. Enteritidis isolates were cultured on Tryptone Soya Agar (TSA, LabM, UK) at 37°C for 24h. The bacterial inoculates were prepared using 18 hours old cultures and suspensions were adjusted to 0.5 McFarland standard turbidity.

Resazurin powder preparation

A stock solution of the resazurin sodium salt (7-Hydroxy-3*H*-phenoxazin-3-one 10-oxide, Himedia,) powder was prepared in sterile distilled water, concentration 0.01%. It was filter-sterilized and kept at 4°C.

Broth microdilution method

Broth microdilution method was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) according to the National Committee for Clinical Laboratory Standards [11], with some modifications. The bacterial inoculates were prepared using overnight cultures and suspensions

were adjusted to 0.5 McFarland standard turbidity.

All tests were performed in MHB (Muller-Hinton Broth, LabM, UK). Propylene glycol (2 - (2 - hydroxypropoxy) - 1-propanol) was used to dissolve the EO and then diluted to the concentration (50 – 0.024 µl/mL). Twenty microliters aliquots of the EO were added to 96-well microtitre plates, in geometric dilutions, ranging from 50 to 0.024 µL/mL. Afterwards, aliquots of 160 µL of MHB, were added into each well. As the final step, 20 µL of $2x10^6$ cfu/mL (according to 0.5 Mc Farland turbidity standards) of standardized bacterial suspensions were inoculated into each microplate. The test was performed in a total volume of 200 µL in each well with final EO concentrations of 5 – 0.0024 µL/mL. Plates were incubated at 37°C for 24 hours. The same tests were performed simultaneously for growth control (MHB + test organism) and sterility control (MHB + test oil).

After 24h of incubation, 20 µL of the resazurin solution (0.01%) was added to each well and the plate was incubated 6h at 37°C. After visual examination the plates were additionally incubated for 18h. A change of color from blue (oxidized) to pink (reduced) indicated the growth of bacteria. The MIC was defined as the lowest concentration of EO that prevented this change in color.

Referring to the results of the MIC assay, the wells showing complete absence of growth were identified and 100 µL solutions from each well was transferred to Plate count agar plates (PCA, LabM, UK) and incubated at 37°C for 24 hours. The MBC was defined as the lowest concentration of the EO at which 99.9% of the inoculated microorganisms were killed.

Inhibition of initial cell attachment

The effect of EO on biofilm formation was evaluated as described by Jadhav, *et al*. [9]. Solutions of EO (equivalent to 0.5×MIC and 1×MIC) were prepared. Twenty microliters of each solution were added to individual wells of a sterile flat-bottomed 96-well polystyrene microtitre plates (Greiner Bio-One, International GmbH). Afterwards, aliquots of 160 µL of TSB were added into each well. As the final step, 20 µL of $2x10^6$ cfu/mL (according to 0.5 Mc Farland turbidity standards) of standardized bacterial suspensions were inoculated into each well to yield a final volume of 200 µL in each well. The cultures were added into the wells in quadruplicate. Control well contained all components except the inoculums (180 µl TSB + 20 µL of specific concentration of EO diluted in propylene glycol). Positive control well contained 160 µl TSB + 20 µL inoculums (in the same broth) + 20 µL of pure propylene glycol; this control is to reveal potential effect of solvent propylene glycol on *Salmonella* growth. Sterility control well contained 180 µL TSB + 20 µL pure propylene glycol; this control is to reveal possible contamination of solvents. The plates were sealed and incubated for 48 hours at 25°C under sterile conditions to allow cell attachment. Biofilm formation was assessed using the modified CV assay.

Inhibition of preformed biofilm

The effect of EO on biofilm growth and development was evaluated as described by Jadhav, *et al*. [9], with some modifications. Biofilms were allowed to be formed for 48h prior to addition of EO. Biofilm formation was achieved by transferring 160 µL of TSB into each microplate, followed by addition of 20 µL of bacterial culture (prepared as described above) into the wells of sterile flat-bottomed 96-well polystyrene microtitre plates in quadruplicates. The scheme of control samples was the same as described in previous section.

The microtitre plates were covered and incubated for 48h at 25°C to allow cell attachment and biofilm formation. Following incubation, 20 µL of each stock solution of EO was added to each well to yield a final volume of 200 µL. After the treatment of preformed biofilms with EO, the plates were incubated for 30 and 60 minutes. Following incubation, the biofilms were assessed for biomass attachment using the modified CV assay.

Biofilm biomass assay (modified CV assay)

Indirect assessment of cell attachment for *S.* Enteritidis was evaluated using the modified CV assay described by Agarwal, *et al*. [12]. Following the 48h incubation (Section Inhibition of initial cell attachment) and (Section Inhibition of preformed biofilm), culture medium from each well was gently removed and the plates were washed three times with 250 µL sterile distilled water to wash away any loosely attached cells. The plates were air dried for 45 min. The cells in the biofilm were then stained with 250 µL 0.3% CV and incubated at room temperature for 15 min. The stain was removed by exhaustive washing with distilled water.

The plates were then allowed to dry. In order to quantify adhered cells, 250 µL of decolouring solution (ethanol/acetone, 80:20%) was added to each well for 15 min. The absorption of the eluted stain was measured at 595 nm using a microplate reader (ChemWel, Awareness Technology). The mean absorbance ($OD_{595\,nm}$) was used for determining the percentage inhibition of biomass formation for each concentration of the oil according to the following equation:

$$\% \text{ inhibition} = 100 - \left[\left(\frac{\text{OD 595 experimental well with components of EO}}{\text{OD 595 control well without components of EO}}\right) \times 100\right]$$

Statistical analyses

Statistical analysis was performed by Statistica 12 (StatSoft Inc., Tulsa, Oklahoma). Due to the size of the sample (n<30), the data from the assays were compared using the nonparametric Mann-Whitney test. Results were considered to be statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

Results for antibacterial activity of *Th. vulgaris* obtained using broth microdilution method against *S.* Enteritidis were identical for all tested isolates (MIC/MBC=0.156/0.3125 µL/mL). Between the tested isolates of *S.* Enteritidis are not established differences in response to treatment with essential oil. This coincides with the findings of Lu and Wu [13] who examined antimicrobial effect of *Th. vulgaris* essential oil, carvacrol and thymol on four different serotype of *Salmonella enterica:* Kentucky, Senftenberg, Enteritidis and Typhimurium. Obtained results for antimicrobial activity of *Th. vulgaris* essential oil are consistent with previous studies [14-17]. These studies reported MIC for serotype Enteritidis that ranged from 0.025 µL/mL [18] to 0.625 µL/mL [16], whereas MBC for these serotype ranged from 0.025 µL/mL [18] to 1.2 µL/mL [15].

Ability of *Salmonella* spp. to form biofilms on different surfaces has been demonstrated in many researches [2, 19, 20].

The CV assay indicated that the effect of *Th. vulgaris* essential oil on initial cell attachment of tested isolates SE4, SE5, SE7 and SE9 was dosage dependent manner, although even at 0.5×MIC biomass attachment was reduced by 74.2%, 77.7%, 73.6% and 70.1%, respectively. Fairly high, but not complete, inhibition of cell attachment was achieved using 1×MIC of this EO. Using 1×MIC, biomass attachment of tested isolates SE4, SE5, SE7 and SE9 was reduced for 91.4%, 90.2%, 90.6% and 88.4%, respectively (Figure 1). These results are consistent with the results of other researchers[21, 22]. Namely, Soni *et al*., [21] determined that sub-lethal concentrations up to 0.012% (approx. 0.12 µL/mL) of carvacrol, a component of thyme and oregano essential oil, significantly reduced biofilm formation in three strains of *S.* Typhimurium. In research of Burt *et al*., [22] significant reduction with carvacrol in *S.* Typhimurium biofilm formation was found between 0.75 mM - 1.25 mM (approx 0.11 - 0.19 µL/mL). Since they determined that the number of viable bacteria was not reduced using sub-lethal concentrations of carvacrol, these authors assumed that biofilm development can be inhibited without reducing bacterial viability, and that a mechanism other than growth inhibition or bacterial cell death may be involved in this antibiofilm activity of carvacrol. More recent studies show that carvacrol reduces bacterial motility at sub-lethal concentrations owing to their ability to interfere with the quorum sensing (QS) signaling mechanism between bacterial cells, thereby also reducing the capacity for biofilm formation [22, 23]. But precise mechanism by which carvacrol inhibits enlargement of biofilm has not yet been fully established.

Figure 1.

Effect of tested concentrations of *Th. vulgaris* essential oil (0.5×MIC and 1×MIC) on pre-formed biofilms of tested isolates *S.* Enteritidis SE4, SE5, SE7 and SE9 is shown in Figure 2 and 3.

Following the first 30 minutes of incubation, preformed biofilm of tested isolates *S.* Enteritidis SE4, SE5, SE7 and SE9 with *Th. vulgaris* essential oil, only 27.8%, 18.5%, 19.4% and 28.1% inhibition occurred at 0.5×MIC level, respectively (Figure 2), while 1×MIC of this EO reduced preformed biofilm for 50.0%, 35.5%, 38.9% and 51.8%, respectively (Figure 3).

Figure 2.

After 60 minutes of incubation with *Th. vulgaris* essential oil with the preformed biofilm of *S.* Enteritidis tested isolates (SE 4, SE5, SE7 and SE9), only 33.7%, 23.8% 22.5% and 38.4% inhibition occurred at 0.5×MIC level, respectively (Figure 2). Likewise, inhibition of biofilm formation at 1×MIC

was slightly higher than at 0.5×MIC, and amounted 54.0%, 40.7%, 41.8% and 51.8%, respectively (Figure 3).

Percent inhibition of preformed biofilms did not rise significantly after periods longer than 30 min ($p<0.05$) (Figure 2 and 3).

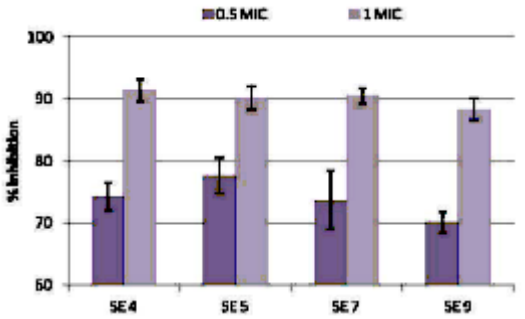

Fig. 1. Effect of different concentrations of *Th. vulgaris* essential oil (expressed as percentage inhibition of biofilm formation) on initial cell attachment of tested isolates *S. enteritidis* (SE4, SE5, SE7 and SE9)

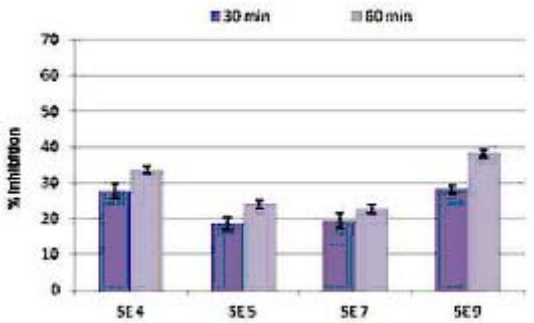

Fig. 2. Effect of 0.5 MIC *Th. vulgaris* essential oil (expressed as percentage inhibition of biofilm formation) on preformed biofilm (48h) of tested isolates *S.* Enteritidis (SE4, SE5, SE7 and SE9)

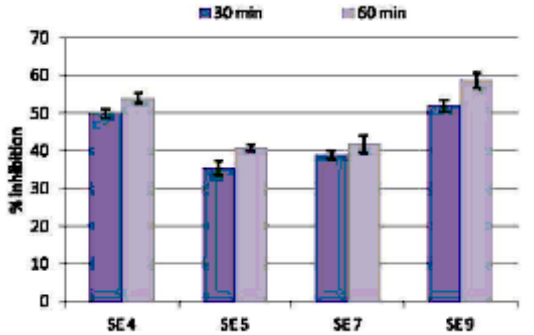

Fig. 3. Effect of 1×MIC *Th. vulgaris* essential oil (expressed as percentage inhibition of biofilm formation) on preformed biofilm (48h) of tested isolates *S. enteritidis* (SE4, SE5, SE7 and SE9)

The ability of biofilm production by isolates applied in this study was previously characterized. Isolates SE4 and SE9 were characterized by moderate biofilm production whereas the phenotype on Congo red agar corresponded with BDAR (brown, dry, and rough) morphotype. SE5 and SE7 isolates were characterized by the strong biofilm production and the phenotype on Congo red agar corresponded with RDAR (red, dry, and rough) morphotype (data not shown). An large number of studies have reported that extracellular matrix component of Salmonella biofilm, cellulose, are an important characteristic for extracellular survival and is directly responsible for resistance to different antimicrobials [24, 25]. We noticed slightly lower percentage of inhibition of the isolates SE5 and SE7 for which we previously established to produce cellulose in biofilm matrix (RDAR morphotype), while the production of cellulose in the matrix of the biofilm was not determined (BDAR morphotype) for the isolates SE4 and SE9.

Overall, the data presented here show that resistance of a preformed biofilm can be associated with the presence of production of extracellular polymeric substance or biofilm matrix. The extracellular matrix limits the penetration of antimicrobial agents into the biofilm. This is partly due to diffusion limitation caused by the 3-dimensional structure, but primarily because of absorption or reaction of the antimicrobial agent with extracellular matrix components. This takes place at the outer part of the biofilm and neutralizes the antimicrobial agent. Therefore the innermost bacterial cells of the biofilm are not reached by the antimicrobial agent and survive the treatment. Another factor which may contribute to this increased resistance is that the majority of antimicrobial compounds are more effective against actively growing cells. The cells in a biofilm have a poor growth rate due to lack of nutrients and oxygen, which may reduce the antimicrobial effects of compounds against them [26].

CONCLUSIONS

Present investigation demonstrated that *Th. vulgaris* essential oil is effective not only on planktonic cells but also on biofilms of *S.* Enteritidis. Overall, essential oil of *Th. vulgaris*

was found to be more effective in inhibiting initial cell attachment compared to preformed biofilms. Due to excellent inhibitory effect of *Th. vulgaris* essential oil on initial cell attachment, the use of this essential oil and its components with a view to preventive inhibition of biofilm formation is a promising approach. Essential oil and its components would allow inclusion of these compounds in novel pharmaceutical products, disinfectant and sanitizer formulations.

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