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Research Article

Antimicrobial effect of thyme essential oil against *Salmonella enteritidis* inoculated in flour and homogenized eggs

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Abstract

The aim of this study was to determine the antimicrobial effect of thyme essential oil against two strains of *Salmonella enteritidis* group D: reference strain ATCC 13076 and epidemical strain. The both strains of bacteria were inoculated separately up to concentration of 10^9 CFU/mL in the micellar solutions which were prepared as application of thyme essential oil in physiological solution up to final concentration of 1; 2.5 and 5%. That stock micellar solutions were used for preparing the mixture with white flour and homogenized eggs, separately. The prepared samples were cultivated on plate and exposed at a temperature of 37°C for 18 hours and at a temperature of 46°C for 9 hours (ISO 6579-1). GLM multivariate statistical model showed that the number of both strains of *Salmonella enteritidis* was significantly influenced by the thyme essential oil concentration, type of media and their interaction.The thyme essential oils might be useful in the control of *Salmonella spp.* in commercially produced food systems as an alternative of the chemical preservatives.

Keywords: antimicrobial effect, thyme essential oil, *Salmonella*, flour, eggs.

Abbreviations:

S. enteritidis RS - *Salmonella enteritidis* reference strain ATCC 13076
S. enteritidis ES - *Salmonella enteritidis* epidemical strain
PhS - physiological solution
CFU- colony forming unit
MIC – minimal inhibitory concentration
Log_{10} - logarithm of x to the base 10
GLM – general linear model

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Introduction

Thymus (*Thymus vulgaris* L.) is the most common species of the genus *Thymus*, belonging to the family *Lamiaceae* (Elshafie and Camele, 2017). Essential oil of thyme has antioxidant and antibacterial properties.

In laboratory conditions, essential oil of thyme showed excellent antibacterial activity against *Escherichia coli* and *Salmonella* spp. (Bosković et al., 2015). Sabulal et al. (2008) have determined the antimicrobial effect of the essential oils extracted from *Thymus vulgaris*, *Thymus zygis* subsp. *gracilis* and *Thymus hyemalis* L. against 10 pathogenic microbial species. Mazzarrino et al. (2015) investigated the antimicrobial effect of 21 essential oils on 10 strains of *Salmonella enteritidis* and *Listeria monocytogenes*. The major antibacterial effects have shown the essential oils of oregano, cinnamon, cloves and red thyme.

Thyme essential oils have become very popular as safe alternative instead chemical preservatives in the context of preventing microbial contamination. Hence, the contributions of the studies that as research objective have antimicrobial effect of thyme essential oils are going in two directions: extending the shelf life of foodstuffs to reduce risk of spoilage and, secondly, minimizing the risk of the presence of pathogenic microorganisms in the final products. For example, Emiroğlu et al. (2010) added thyme and oregano essential oils to increase antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas* spp. and coliform bacteria in beef pate when refrigerated at maintenance temperature. Kostaki et al. (2009) described a procedure for using thyme oil in combination with a mixture of CO_2, N_2 and O_2 in two different ratios, in order to preserve the quality and shelf life of fresh fillet sea bass during storage for a period of 21 days in the refrigerator.

Jemaa et al. (2017) investigated the antimicrobial effect of *Thymus capitatus* essential oil and its nanoemulsion on the milk quality inoculated with *Staphylococcus aureus*. It has been found to have a nearly twice as good antimicrobial effect in the treatment of milk with nanoemulsion than essential oil.

The aim of the study was to determine the antimicrobial effect of different concentrations of thyme essential oils on the growth and reproduction of two *Salmonella* strains (*Salmonella enteritidis* reference strain ATCC 13076 and *Salmonella enteritidis* epidemical strain) in laboratory conditions, inoculated in flour and egg from chicken, as raw material for preparing the dough for making pasta with eggs.

Materials and Methods

The test samples were prepared as solutions of thyme essential oil in physiological solution (PhS) up to final concentration of 1%, 2.5% and 5% (micellar solution), as well as PhS without essential oil, and were inoculated with one of the bacterial strains tested: *Salmonella enteritidis* reference strain ATCC 13076 (*S. enteritidis* RS) and *Salmonella enteritidis* epidemical strain (*S. enteritidis* ES); a mixture of flour "mixed up" with the micellar solutions of thyme essential oil and a mixture of egg previously homogenized with PhS and added micellar solutions of thyme essential oil at different concentrations have been inoculated particularly with the bacterial strains (*S. enteritidis* RS and *S. enteritidis* ES). The control samples were prepared as dip application of bacteria in physiological solution and in inoculums with white flour and eggs without added essential oil.

In order to compare bacterial growth, two types of samples were used: control samples - inoculums of the tested bacterial strains in PhS and in mixture of flour and eggs without micellar solution of thyme essential oil and target samples - samples of flour and eggs "mixed up" with micellar solutions of thyme essential oil and inoculated with each of the tested bacterial strains.

All samples were prepared in duplicate. In all samples were added 90 ml Salenit F broth and one sample was exposed at a temperature of 46 °C (pasta drying temperature) for 9 hours, and the other one was exposed at 37 °C (incubation temperature). Then, the samples were cultivated on plate for enumeration according ISO 6579-1 (2017).

Fresh thyme essential oil solutions (Fitofarm, Skopje) were prepared for each phase of the experiment at concentrations of 1%, 2.5% and 5%, which were used as "micellar solutions" for inoculation with bacteria for "mixing" flour and for homogenizing the egg.

The ready suspension from *S. enteritidis* RS and *S. enteritidis* ES have been inoculated in 5 mL of the micellar solutions of thyme essential oil (1%, 2.5% and 5%) as well as 5 mL of PhS (control), in initial concentration of bacteria from 10^9 CFU/mL

Mixtures of flour were prepared as follows: 9 g of flour and 5 mL of each micellar solution of thyme essential oil in different concentrations (1%, 2.5% and 5%), as well as 9 g of flour and 5 mL of micellar solutions inoculated with bacterial strains.

Chicken egg mixtures were prepared as follows: 5 mL of diluted egg (1 egg with 130 mL of PhS), 5 mL from each micellar solution of thyme essential oil in different concentrations (1%, 2.5% and 5%), as well as 5 mL of diluted egg and 5 mL of micellar solutions inoculated with bacterial strains.

After homogenization of the flour and egg samples, 90 mL of Salenit F broth (Merck KGaA, Germany) were added to each of them, prepared according to the manufacturer's instructions, and the entire contents were vortexed to macroscopically visible homogeneity (vortex- Fisher Bioblock Scientific).

Dilutions 1:20 and 1: 200 were prepared from all samples and from them 0.1 mL was inoculated on Müller-*Hinton agar* (Merck KGaA, Germany), for enumeration of bacterial cell count (CFU). Petri plates were incubated at 37 °C (incubator - Boxun B, Shanghai Boxun Industry and Commerce Co Ltd) for 18 hours (ISO 6579-1. 2017).

Each control and target samples procedure was previously validated in three independent successive experiments, by calculating the mean values used for statistical calculations.

Data analysis was carried out with GLM-General Linear Model testing the null hypothesis – the statistically significant influence of the independent (factor) variables and their interaction on the mean values of the different grouping from the Log_{10} number of bacterial cells of *S. enteritidis* RS and *S. enteritidis* ES.

Results and Discussion

Table 1 shows the results for bacterial cell counts of *S. enteritidis* RS in flour and eggs, with a thyme essential oil solution of different concentrations, following a sample of all samples previously exposed to a temperature of 46 °C for 9 hours (pasta drying process in the manufacturing process), and all samples cultured in laboratory conditions, at an incubation temperature of 37 °C for 18 hours.

Table 1. Number of bacterial cells of *S. enteritidis* RS (Log_{10}) in flour and in eggs medium with micellar solution of thyme essential oil

Combination of micellar solution and medium	t (°C)	n	Control	Concentrations of thyme essential oil		
				1%	2.5%	5%
			($\overline{x} \pm S_{\overline{x}}$)	($\overline{x} \pm S_{\overline{x}}$)	($\overline{x} \pm S_{\overline{x}}$)	($\overline{x} \pm S_{\overline{x}}$)
Flour with micellar solution of thyme essential oil	37 °C	1	5.96	5.58	5.58	5.48
	46 °C	1	5.97	5.53	5.53	5.38
Eggs with micellar solution of thyme essential oil	37 °C	1	5.75	3.60	3.60	0.00
	46 °C	1	5.56	0.00	0.00	0.00
Overall	37 °C	3	5.74±0.130	3.06±1.633	3.06±1.633	1.83±1.826
	46 °C	3	5.66±0.158	1.84±1.843	1.84±1.843	1.79±1.793

The bacterial cell count of *S. enteritidis* RS in control samples of flour without a micellar solution of thyme essential oil, regardless to the exposure temperature, was ranged from 5.96 to 5.97 Log_{10}. In

egg samples these values were slightly lower and ranged from 5.56 to 5.75 Log_{10}. Regardless from the medium, the bacterial cell count of *S. enteritidis* RS was 5.74 ± 0.130 Log_{10} at 37 °C and 5.66 ± 0.158 Log_{10} at 46 °C for 9 hours. In the target samples of flour medium with micellar solution of thyme essential oil, the CFU of *S. enteritidis* RS showed a trend of decrease from the samples with added 1% micellar solution to the samples with 5% micellar solution of thyme essential oil, thereby the slightly greater decrease was recorded in the samples exposed to 46 °C (5.38 Log_{10}) compared to samples exposed to 37 °C (5.48 Log_{10}). There was observed decrease in the CFU of *S. enteritidis* RS in the samples with eggs medium containing 1% and 2.5% micellar solution of thyme essential oil at 37 ° C, while in the samples with 5% micellar solution of thyme essential, regardless the exposure temperature, wasn't find any grouth of *S. enteritidis* RS.

Independently from the inoculated medium, the largest reduction in bacterial cell count of *S. enteritidis* RS was observed in the target samples exposed to 46 °C compared to samples exposed at 37 °C.

According to research done by Millezi et al. (2011), the minimum inhibitory concentration of thyme essential oil (*T. vulgaris*) to *P. aeruginosa* and *S. enteritidis*, was 5% and 10%, respectively. According to Thanissery and Smith (2014), the combination of 0.5% thyme and orange essential oils inhibit the growth of *Salmonella* and *Campylobacter* bacteria. Even when thyme essential oil was added in much smaller portions (0.1%) in chopped lamb packaged in a modified atmosphere, as tested by Karabagias et al. (2011), an antimicrobial effect was found resulting in a significant extension of the shelf life.

Table 2 shows the results for the bacterial cell count of *S. enteritidis* ES in flour and in eggs medium with added a micellar solution of thyme essential oil of different concentrations, as it was described in the methodology.

Table 2. Number of bacterial cells of *S. enteritidis* ES (Log_{10}) in flour and in eggs medium with micellar solution of thyme essential oil

Combination of micellar solution and medium	t (°C)	n	Control	Concentrations on thyme essential oil		
				1%	2.5%	5%
			($\bar{x} \pm S_{\bar{x}}$)	($\bar{x} \pm S_{\bar{x}}$)	($\bar{x} \pm S_{\bar{x}}$)	($\bar{x} \pm S_{\bar{x}}$)
Flour with micellar solution of thyme essential oil	37 °C	1	6.00	5.64	5.70	5.60
	46 °C	1	6.04	5.60	5.85	5.60
Eggs with micellar solution of thyme essential oil	37 °C	1	5.60	0.00	4.00	0.00
	46 °C	1	5.56	0.00	3.78	0.00
Overall	37 °C	3	5.70±0.150	1.88±1.880	3.23±1.689	1.86±1.866
	46 °C	3	5.76±0.142	1.86±1.866	3.21±1.712	1.86±1.866

The bacterial cell count of *S. enteritidis* ES in samples of flour without a micellar solution of thyme essential oil, regardless of the exposure temperature, was ranged from 6.00 to 6.04 Log_{10}, while in the samples with eggs these values were slightly lower and were ranged from 5.56 to 5.60 Log_{10}.

Independently from the inoculated medium, the average bacterial cell count of *S. enteritidis* ES in the control samples was varied from 5.70 ± 0.150 Log_{10} at 37 °C to 5.76 ± 0.142 Log_{10} at 46 °C. There was recorded decreasing in the bacterial cell count of *S. enteritidis* ES in all target flour samples, regardless to concentration of thyme essential oil and exposure temperature, with the highest evidented decrease in the target samples with added 5% micellar solution of thyme essential oil. There wasn't evidented any grouth of *S. enteritidis* ES in the target eggs containing samples with added 1% and 5% micellar solution of thyme essential oil,

keeping the established decreasing trend in CFU comparing to control samples. However, there was decreasing trend of CFU in the samples with added 2.5% micellar solution of thyme essential compared to control samples, but steel there was recorded grouth of *S. enteritidis* ES. regardless of the temperature exposure.

Independently to the inoculated medium, the largest decrease in bacterial cell count of *S. enteritidis* ES was observed in target samples with added micellar solution of thyme essential oil in concentration of 1% and 5%.

Bajpai et al. (2012) indicate that the MIC of thyme essential oil for *S. typhimurium* inhibition has a wide range of 0.45 to 720 µL / mL, whereas for *S. enteritidis* even higher , ranged from 66.7 to 320 µg / mL. Similar values for MIC of thyme essential oil related to six salmonella serovars were obtained by Bošković (2016). Thus, according to this author, growth inhibition of *S. enteritidis* and *S. typhimurium* requires at least 320 µg / mL thyme essential oil, whereas inhibition of *S. montevideo*, *S. senftenberg*, *S. infantis*, and *S. givae* requires twice as much concentration 640 µg / mL. With regard to the active compounds in thyme essential oil, thymol was found to have the best antimicrobial effect, with the minimum inhibitory concentration for *S. enteritidis* being 160 µg / mL, and for *S. montevideo*, *S. senftenberg*, *S. infantis* and *S. givae*, this value was twice as high (320 µg / mL). Oposite Oulkheir et al. (2017) were determinate MIC of thyme essential oil ranged from 5 to 10 mg / mL.

Table 3 shows the results from the multivariate general linear model for the effect of thyme essential oils on the bacterial counts of *S. enteritidis* RS and *S. enteritidis* ES in the media used in the study (physiological solution / micellar solution, flour and eggs), cultivated in the laboratory.

Table 3. Influence of fixed variables and their interaction on bacterial cell counts of *S. enteritidis* RS and *S. enteritidis* ES in micellar solution, flour and eggs based media

Fixed variables	df	*S. enteritidis* RS + thyme essential oil	*S. enteritidis* ES + thyme essential oil
Model[a,b]	11	13.390***	3486.047***
Overall average	1	213.563***	51908.697***
Concentration	3	17.120***	4277.001***
Media	2	36.933***	8971.616***
Concentration x media	6	3.676*	1262.083**
Error	12	1.082	0.005
Total	24		
[a]R^2 = 0.829; [b]R^2 = 0.965;			

*** statistically significant at the level p < 0.001
** statistically significant at the level p < 0.01
* statistically significant at the level p < 0.05

Performed statistical models showed that there was a significant influence of thyme essential oil and used medium on CFU of *S. enteritidis* RS and *S. enteritidis* ES at level p<0.001, while their interaction has statistically significant influence at level p<0.05 for CFU of *S. enteritidis* RS and at level p <0.01 for CFU of *S. enteritidis* ES.

The value for R^2 in both statistical models was high. This means that most of the variance in CFU of *S. enteritidis* RS and *S. enteritidis* ES laboratory cultivated according to the described methodology can be explained by the fixed factors used in the study.

In the context of the results obtained from the study, it should be mentioned that a large number of authors were interested in testing the antimicrobial effect of thyme essential oil on pathogenic bacteria in food from animal origin, such as eggs, meat, milk and milk products. All of them revealed that there was an antimicrobial effect on thyme essential oil. Thus, for example, Boskovic (2016) found a statistically significant decrease in the initial

number of bacterial cells from more bacterial species of the genus *Salmonella* (10^6 = 6 Log cfu / g) inoculated into minced pork with added thyme essential oil in concentrations from 0.3%, 0.6% and 0.9%. The meat samples were packed in vacuum and in modified atmosphere (30% O_2 / 50% CO_2 / 20% N_2). All samples were stored at 3 ± 1 ° C. The decrease in the number of bacterial cells increased proportionally with the increasing of the essential oil concentration (0.3%, 0.6% and 0.9%). Herewith, we couldn't exclude the influence of modified atmosphere and storage temperature on bacterial cell reduction The highest reduction in *Salmonella* bacteria cell counts was recorded on the ninth and third day after the addition of thyme essential oil.

Conclusions

The thyme essential oils might be useful in the control of *Salmonella spp.* in commercially produced food systems as an alternative of the chemical preservatives. The bacterial cell count of *S. enteritidis RS* and *S. enteritidis* ES in the flour and egg samples was gradually reduced depending from the thyme essential oil concentration combine with temperature exposed.

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