



Research Article

Antimicrobial effect of sweet basil essential oil against *Salmonella enteritidis* growth *in vitro*

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Abstract

The aim of the study was to examine the antimicrobial activity of sweet basil 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 10⁹ CFU/mL in the micellar solutions which were prepared as application of sweet basil 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. In all samples was added 90 mL Salenit F broth and were exposed at a temperature of 37°C for 18 hours and at a temperature of 46°C for 9 hours. The samples were cultivated on plate for enumeration according ISO 6579-1. The results from the GLM multivariate statistical model indicate that the number of both strains of *Salmonella enteritidis* cultivated on plate was high significantly influenced by the concentration of the sweet basil essential oil, medium and also their interaction. These results support the possibility of using sweet basil essential oil as natural preservative in food like pasta, prepared from white flour and eggs.

Keywords: antimicrobial effect, sweet basil essential oil, *Salmonella*, *in vitro*.

Abbreviations:

S. enteritidis RS - *Salmonella enteritidis* reference strain ATCC 13076

S. enteritidis ES - *Salmonella enteritidis* epidemical strain

PhS - physiological solution

CFU- colony forming unit

Log₁₀ - logarithm of x to the base 10

GLM – general linear model

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Introduction

Recently, worldwide the use of synthetic preservatives in food is increasingly avoided because some of them have been found to have toxic and harmful effects on consumer health and the environment (Chivandi et al. 2016). On the other hand, the emergence of resistance to antimicrobials is one of the major threats to public health (López-Pueyo et al. 2011). Hence, the use of natural antibacterial essential oils as a substitute for synthetic antimicrobials is of great importance as most of them have proven efficacy. They are considered a safe and environmentally friendly alternative to the control of bacteria present in the food and food industry, but also to the control of other pathogenic microorganisms, especially those that are drug resistant (Friedman et al. 2002; Yap et al. 2013, 2014).

Basil (*Ocimum basilicum* L.) essential oil has a pronounced antioxidant, antimicrobial, antihypertensive, anticancer and antiinflammatory effect (Arranz et al. 2015). Estragol and linalool are the main antimicrobial compounds of basil essential oil (Elansary et al. 2016; Mith et al. 2016; Avetisyan et al. 2017; Hanif et al. 2017). In the research carried out by Moghaddam et al. (2011), basil essential oil showed a better effect on gram-negative than gram-positive bacteria. According to Fyfe et al. (1998), 0.2% basil oil is a potent inhibitor of *Salmonella enteritidis* and *Listeria monocytogenes*. Mint and basil oils at a concentration of 0.08 mL / L had an adverse effect on the survival of *Escherichia coli* and *Salmonella typhimurium*, inoculated in raw chopped salad, as well as during its refrigerated storage (Karagözlü et al. 2011). According to Shirazi et al. (2014) the MIC value for basil essential oil for gram-negative *Salmonella typhi* and *Escherichia coli* was 145 - 160 µg / mL.

The main objective of the study was to determine the antimicrobial effect of different concentrations of basil essential oil on the growth and reproduction of two strains of *Salmonella* (*Salmonella enteritidis* reference strain ATCC 13076 and *Salmonella enteritidis* epidemical strain) in laboratory conditions, inoculated in flour and egg from chicken, which are used as raw materials for the

preparation of the dough for making the pasta with eggs.

Materials and Methods

The test samples were prepared as solutions of basil 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 each 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 basil essential oil and a mixture of egg previously homogenized with PhS and added micellar solutions of basil essential oil at different concentrations have been inoculated with the both bacterial strains (*S. enteritidis* RS and *S. enteritidis*). 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 basil essential oil and target samples - samples of flour and eggs "mixed up" with micellar solutions of basil essential oil and inoculated with each of the tested bacterial strains.

All samples were prepared in two repetitions. In all samples were added 90 mL Salenit F broth and one sample was exposed at a temperature of 46°C (pasta drying temperature to control the influence of temperature on the pasta drying process) 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 basil 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 basil essential oil (1%, 2.5% and 5%) as well as 5 mL of PhS (control), in initial concentration of bacteria from 10^9 CFU.

Mixtures of flour were prepared as follows: 9 g of flour and 5 mL of each micellar solution of basil 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 basil 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 - statistically significant influence of the independent (factor) variables and their interaction on the mean values of the different grouping from the number of bacterial cells of *S. enteritidis* RS and *S. enteritidis* ES expressed as Log_{10} number.

Results and Discussion

In table 1 are shown the results for the bacterial cell count of *S. enteritidis* RS in flour and in eggs prepared according to the described methodology.

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

Combination of micellar solution and medium	t (°C)	n	Control ($\bar{x} \pm S_{\bar{x}}$)	Concentrations of basil essential oil		
				1% ($\bar{x} \pm S_{\bar{x}}$)	2.5% ($\bar{x} \pm S_{\bar{x}}$)	5% ($\bar{x} \pm S_{\bar{x}}$)
Flour with micellar solution of basil essential oil	37°C	1	5.98	5.70	5.85	5.60
	46°C	1	5.95	5.75	5.85	5.60
Eggs with micellar solution of basil essential oil	37°C	1	5.56	5.60	5.53	5.30
	46°C	1	5.53	5.60	5.48	0.00
Overall	37°C	3	5.69±0.145	3.76±1.88	3.79±1.898	3.63±1.818
	46°C	3	5.59±0.190	3.78±1.892	3.77±1.891	1.86±1.866

The control samples showed similar values for the CFU of *S. enteritidis* RS in the flour and egg based medium, regardless of the exposure temperature, ranged from 5.95 to 5.98 Log_{10} in the flour medium and from 5.53 to 5.56 Log_{10} in egg medium. Regardless the medium, the CFU of *S. enteritidis* RS was $5.59 \pm 0.190 \text{Log}_{10}$ at 46°C and 5.69 ± 0.145

Log_{10} at 37°C. In the target samples in flour and eggs medium, the CFU of *S. enteritidis* RS decreased gradually from the samples with added micellar solution of basil essential oil in a concentration of 1% to the the samples with added micellar solution in the concentration of 5% ($3.63 \pm 1.818 \text{Log}_{10}$ and $1.86 \pm 1.866 \text{Log}_{10}$, respectively



for exposure at temperature from 37°C and 46°C. There wasn't find any growth of *S. enteritidis* RS in the target samples with eggs and micellar solution of basil essential oil in a proportion of 5%, exposed to 46°C for 9 hours.

Table 2 shows the results for the number of bacterial cells of *S. enteritidis* ES in flour and eggs with a micellar solution of basil essential oil with different concentrations, cultivated on nutrient media and exposed at 37°C and 46°C.

Table 2. Number of bacterial cells of *S. enteritidis* ES (Log₁₀) in flour and eggs medium with micellar solution of basil essential oil

Combination of micellar solution and medium	t (°C)	n	Control ($\bar{x} \pm S_{\bar{x}}$)	Concentrations on basil essential oil		
				1% ($\bar{x} \pm S_{\bar{x}}$)	2.5% ($\bar{x} \pm S_{\bar{x}}$)	5% ($\bar{x} \pm S_{\bar{x}}$)
Flour with micellar solution of basil essential oil	37°C	1	6.00	5.70	5.85	5.72
	46°C	1	6.04	5.78	5.90	5.78
Eggs with micellar solution of basil essential oil	37°C	1	5.60	5.60	5.58	5.60
	46°C	1	5.56	5.70	5.56	3.30
Overall	37°C	3	5.70±0.150	3.76±1.883	3.81±1.906	3.77±1.881
	46°C	3	5.76±0.142	3.82±1.913	3.82±1.912	3.02±1.674

The bacterial cell count of *S. enteritidis* ES in control samples with flour regardless of the exposure temperature, was ranged from 6.00 to 6.04 Log₁₀. In egg samples these values were slightly lower and ranged from 5.56 to 5.60 Log₁₀. Regardless of the medium, the bacterial cell count of *S. enteritidis* ES in control samples was 5.70 ± 0.150 Log₁₀ at 37°C and 5.76 ± 0.142 Log₁₀ at 46°C. In the target samples with flour and eggs based mediums, the number of bacterial cells of *S. enteritidis* ES was gradually reduced from the samples with added 1% micellar solution of basil essential oil to the samples with 5% micellar solution of basil essential oil, when the lowest growth was registered (3.77 ± 1.881 Log₁₀ at 37°C and 3.02 ± 1.674 Log₁₀ at 46°C).

Exceptions from this trend showed the eggs samples with micellar solution of basil essential oil exposed to 37°C. There wasn't registered any decrease in CFU regarding the basil essential oil concentration and compared to control samples. However, in samples of eggs with 5% micellar solution of basil essential oil exposed to a temperature of 46°C for 9 hours, a greater decrease in the bacterial cell count of *S. enteritidis* ES compared to control samples was observed (3.30 Log₁₀ compared to 5.56 Log₁₀).

Herewith, the findings of some authors suggest that the reason for the weaker antimicrobial effect of plant essential oils, including basil essential oil, is the interaction that have essential oils and bacteria with the food ingredients (Burt 2004; Hayouni et al. 2008). Proteins bind phenolic compounds, while essential oils dissolve in the lipid fraction due to their hydrophobic nature, making them less likely to interact with bacteria (Hayouni et al. 2008). Food fats, on the other hand, form a protective layer around the bacterial cell and thus protect it from the action of antimicrobials (Cutter 2000). Therefore, according to some of these studies, it has been found that in order to obtain the optimal antibacterial effect in the food (bacteriocidal and bacteriostatic), it is necessary to use several times higher concentration of essential oils of plants than the laboratory determined concentration, expressed as MIC (minimum inhibitory concentration) or MBC (minimum bactericidal concentration) (Shelef et al. 1984; Hayouni et al. 2008; de Oliveira et al. 2013). Table 3 presents the results from the multivariate general linear model for influence of the effect of basil essential oil on the bacterial cell counts of *S. enteritidis* RS and *S. enteritidis* ES in the media under 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	<i>S. enteritidis</i> RS + basil essential oil	<i>S. enteritidis</i> ES + basil essential oil
Model ^{a,b}	11	11.139***	58.482***
Overall average	1	325.417***	1886.370***
Concentration	3	7.424**	29.652***
Media	2	37.067***	211.297***
Concentration x media	6	4.352*	21.957**
Error	12	1.172	0.223
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 on CFU of *S. enteritidis* RS and *S. enteritidis* ES at level $p < 0.001$ for basil essential oil concentration and the medium, while their interaction have 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. Exceptionally, the concentration of basil essential oil has statistical significant influence on level $p < 0.01$ on reduction of CFU of *S. enteritidis* RS.

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.

The results obtained from the laboratory research for determination of the antimicrobial effect of basil essential oil are similar with other literature data. Thus, according to the research done by [Rattanachaikunsopun and Phumkhachorn \(2010\)](#), basil essential oil (*Ocimum basilicum*) has shown a high antimicrobial effect on *Salmonella enteritidis*. [Nabrdalik and Grata \(2016\)](#) investigated the effect of basil essential oil added at different concentrations (0.25, 0.5, 1.0, 2.0 and 4.0%) on *Salmonella enteritidis* in a nutrient broth supplemented with 0.05% (v / v) Tween 80

(polyethylene sorbitol ester). The initial number of bacterial cells was 10^8 cfu/mL (8 log cfu/mL). The samples were exposed to a temperature of 37 °C for 4, 24, 48 and 168 h. The reduction in the bacterial cell count of *Salmonella enteritidis* ranged from 3 to 26% at 4 h exposure, to 22 - 46% at one week exposure. In absolute terms, the initial bacterial count of 10^8 cfu / mL (8 log cfu / mL) after 24-hour exposure decreased to 6.0287, 5.8783, 5.5903, 5.6037 and 5.6007 log cfu / mL corresponding to the concentrations of basil essential oil used (0.25, 0.5, 1.0, 2.0 and 4.0%), which was statistically significant at $p < 0.05$. These results are relatively similar to those obtained in our study when the temperature treatment of the control samples with egg mixture inoculated with *Salmonella* strains evoked reduction in CFU ranged from 37.78 - 38.55%.

The target samples of egg mixtures with micellar solution of basil essential oil had a 100% antimicrobial effect on *S. enteritidis* RS, whereas the bacterial cell count of *S. enteritidis* ES decreased by 63.33% in ratio of the initial bacterial cell count (from 9 Log₁₀ to 3.3 Log₁₀), at an exposure temperature of 46°C. The greater antimicrobial effect in target samples of egg mixtures with micellar solution of basil essential oil than in samples of flour mixture, may be explained by the



influence of the antimicrobial substances present in the eggs as the flour doesn't contain any antimicrobial substances. Some literary data support this conclusion. Many proteins, such as cystatin (Saxena and Tayyab 1997; Werierska et al. 1991), ovomacroglobulin (Miyagawa et al. 1991) and avidin (Board and Fuller 1974), are associated with the antimicrobial effect of chicken egg (*Gallus gallus*). Ovotransferin, the main egg white protein, consists of two receptors, each of them reversibly binds iron, limiting its amount and thereby inhibiting the growth of microorganisms (Seviour and Board 1972; Phelps and Antonini 1975; Valenti et al. 1981). Another important component of egg protein is lysozyme (N-acetylmuramide-glycanhydrolase). It is an enzyme that breaks down the link between glycosidic β -1,4-linked residues of N-acetylneuraminic acid (NAN) and N-acetylglucosamine (NAG) in the structure of peptidoglycan of Gram-positive bacteria and in some cases of Gram-negative bacteria (Burley and Vadehra 1989; Bera et al. 2005).

Conclusions

The bacterial cell count of *S. enteritidis* RS and *S. enteritidis* ES in the flour and egg samples was gradually reduced depending from the basil essential oil concentration, medium and also their interaction. The GLM multivariate statistical model indicate that Log_{10} CFU number of *S. enteritidis* RS and *S. enteritidis* ES cultivated on plate was statistically high significantly influenced by the concentration of the sweet basil essential oil, than by the type of medium and also their interaction. These results support the possibility of using sweet basil essential oil as natural preservative in food like pasta, prepared from white flour and eggs.

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