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Original Research

Antibacterial activity of some Lamiaceae species against *Staphylococcus aureus* in yoghurt-based drink (Doogh)

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Abstract: Doogh is a dairy drinkable fermented product, whose shelf-life and quality is mostly affected by bacteria such as *Staphylococcus* spp.. This study investigated the antibacterial activity of essential oils (EOs) from *Thymus vulgaris* L., *Mentha piperita* L. and *Ziziphora tenuior* L., alone or in combination, against *Staphylococcus aureus* in industrial doogh. A three-level and three-variable face centered central composite design experiment was used. Results showed that EOs significantly inhibited *S. aureus* growth after 1 and 7 days of storage. According to the model, the maximum inhibition was obtained in the presence of 0.2% of EO, independently of the type, and no synergistic or additive effects were observed. Slightly lower *S. aureus* survivals were observed at the maximum concentration of *Z. tenuior* EO. In spite of the antimicrobial activity of these EOs, further research is needed to assess their performance in food matrix and, in particular, in dairy product.

Key words: Antimicrobial activity; *Thymus vulgaris* L.; Peppermint; *Ziziphora tenuior* L.; Response surface methodology; Face centered central composite design.

Introduction

Doogh is a dairy drink produced from yogurt, adding water, salt and other ingredients (*i.e.* natural plant essentials oils). Indian has produced it for the first time, under the name of Lassi. Nowadays, this beverage is used in some countries like Iran and Turkey. It is a healthy dairy drink with pleasant organoleptic notes, which can appropriately substitute for soft drink in all Iranian's food baskets. Its annual production reached 3000000 tons in 2010 in Iran for domestic consumption (1). Milk and dairy products, including doogh, are frequently associated with the presence of *Staphylococcus aureus*, which is considered a dangerous threat to the safety of fermented milk and fresh or low-ripened cheeses (2-4).

Staphylococci are bacteria ubiquitously distributed in nature and commonly isolated from many food products and environmental samples (5). Staphylococci grow over a wide range of temperatures (between 7 and 48 °C) and pH values (between 4 and 10) and are particularly resistant to NaCl (10-15%) and to several antibiotics (3). Moreover, coagulase positive staphylococci show pathogenic traits. In particular, *S. aureus* can cause food poisonings, producing a wide variety of enterotoxins responsible for staphylococcal food poisoning syndrome in humans (nausea, vomiting and abdominal cramps) (3, 6-10).

Plants and their parts are greatly used in traditional healing systems; only in some cases, their therapeutic potential in human has been substantiated (11-21). The need of herb-based medicines, food supplements, cosmetics, pharmaceuticals and health products is gradually increasing throughout the world (12, 22-34). The Lamiaceae family includes 212 genuses and 5600 species and comprises various herbaceous, ornamental and edible plants, some of which are considered as source of essential oils (EOs) with strong antibacterial and antioxidant properties. This family has been used in traditional medicine since earlier times and usually used as a remedy for gastrointestinal tract infection (11). EOs are substances naturally synthesized in different plant organs and can be extracted to be used as complementary medicine, natural therapeutic and food preservatives for their antimicrobial and antioxidant properties (23, 35-37). Some species belonging to Lamiaceae family, such as *Thymus vulgaris* L., *Ziziphora tenuior* L. and *Mentha piperita*, are well-known as aromatic and medicinal herbs. Moreover, the antimicrobial effects of Lamiaceae EOs and their main components (such as carvacrol and thymol) have been reported against a huge variety of Gram-positive (among which food-borne pathogens such as *Staphylococcus aureus*) and Gram-negative bacteria, yeasts and moulds (38-46). The antimicrobial activity of these EOs is promising also in dairy products (47-51).

However, despite the demonstrated potential of EOs and their constituents *in vitro*, their efficacy in food systems may be influenced by several important variables (*i.e.* concentration and solubility, method of extraction, interaction with food matrix, pH, temperature, contamination level, etc.). Therefore, their use has been limited because of the high concentration needed to achieve sufficient antimicrobial activity (52). Although limited studies have been conducted on the antimicrobial interaction between more than two EOs, some synergistic effects of these substances are known and can be useful to reduce the amounts added to food, limiting their organoleptic impact on the products (53). In this perspective, response surface methodology (RSM) method can be used for the evaluation of the effects of multiple variables and their interaction on a response variable (*i.e.* bacterial growth), limiting the number of experiments (54, 55).

In this study, the antibacterial activities of *T. vulgaris* L., *M. piperita* and *Z. tenuior* L. EOs against *S. aureus* in doogh were evaluated. In particular, the survival of two *S. aureus* strains, deliberately inoculated into the doogh samples, was studied after 24 hour and 7 days of refrigerate storage and the results were evaluated with RSM.

Materials and Methods

Staphylococcus aureus suspension preparation

Microbial strains of *S. aureus* (ATCC 33591; PTCC 1764) were provided as lyophilized vials from the Inflectional and Industrial Fungi and Bacteria collection Center in Biotechnology unit of Iranian Research Organization for Science and Technology (Mashhad, Iran). Before the experiments, the strains were cultured twice in nutrient broth (Merck, Germany) at 37 °C for 24 h and plated onto mannitol salt agar medium (Merck, Germany), incubated at the same conditions. The colonies were used to obtain a 0.5 standard microbial suspension using McFarland procedure (56). For this procedure, the 0.5 standard suspension was produced by mixing slowly 99.5 ml of 1% sulfuric acid (H_2SO_4) and 0.5 ml of 1.175% barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$). The intensity of cell suspension density was measured at 625 nm by spectrophotometer (SIGMA-3-30K) in order to have a cell concentration of about 8 log CFU/ml.

Essential oil preparation

The prepared food grade EOs of common thyme (*T. vulgaris* L.), peppermint (*M. piperita*) and *Z. tenuior* L. were purchased from company of Barij Essence Kashan (Isfahan, Iran) in liquid form. The EOs have been stored in dark glass bottle at 4°C to prevent the negative effect of environmental conditions such as direct sunlight until analyses.

Table 1. Independent variables and their coded values used in the face centered composite design.

Independent variable	Symbol	Coded level -1	0	+1
Concentrations of *T. vulgaris* EO (%v/v)	A	0	0.1	0.2
Concentrations of *M. piperita* EO (%v/v)	B	0	0.1	0.2
Concentrations of *Z. tenuior* EO (%v/v)	C	0	0.1	0.2

Experimental design

A face centered central composite design (CCF) with 3 variables (*T. vulgaris* L., *M. piperita* L. and *Z. tenuior* L. concentration) at 3 levels was used as experimental design. The variables and levels used in the twenty experimental runs (including six replicates at the center point) are reported in Table 1 and Table 2.

Preparation of doogh samples containing EOs

The provided doogh samples were purchased from local market in Zabol, Sistan and Baluchestan province of Iran. The doogh were prepared and analyzed by the Food Quality Control laboratory of Zabol University of Medical Sciences, Zabol, Iran. In particular, the doogh was sterilized in autoclave and poured in 20 tubes of

Table 2. Experimental design adopted for the evaluation of the survival of *S. aureus* in doogh samples and observed response values (log CFU/ml) for each run in relation to EO concentrations.

Run	*T. vulgaris* (A, %v/v)	*M. piperita* (B, %v/v)	*Z. tenuior* (C, %v/v)	Dependent variable (log CFU/ml): *S. aureus* counts at 24h	*S. aureus* counts at 7 days
1	0.00	0.00	0.00	5.09	4.91
2	0.20	0.00	0.00	3.00	1.90
3	0.00	0.20	0.00	2.94	1.85
4	0.20	0.20	0.00	2.99	1.87
5	0.00	0.00	0.20	2.56	1.45
6	0.20	0.00	0.20	2.94	1.82
7	0.00	0.20	0.20	2.38	1.27
8	0.20	0.20	0.20	2.85	1.75
9	0.00	0.10	0.10	2.87	2.12
10	0.20	0.10	0.10	3.04	2.27
11	0.10	0.00	0.10	3.14	2.06
12	0.10	0.20	0.10	3.10	1.97
13	0.10	0.10	0.00	3.48	2.54
14	0.10	0.10	0.20	3.08	2.22
15	0.10	0.10	0.10	3.15	2.18
16	0.10	0.10	0.10	3.04	2.08
17	0.10	0.10	0.10	3.12	2.13
18	0.10	0.10	0.10	2.99	2.02
19	0.10	0.10	0.10	2.95	2.06
20	0.10	0.10	0.10	3.15	2.21

Table 3. Parameters estimated for the final polynomial equation model.

Time	Intercept	A	B	C	AB	AC	BC
24 h	5.30	-10.35	-10.55	-12.61	38.50	46.50	34.00
		R^2= 0.801	$F\text{-test}_{(6,13)}$= 8.725 (p=0.000604)				
168 h	4.36	-10.71	-10.90	-12.95	39.13	48.13	35.63
		R^2= 0.803	$F\text{-test}_{(6,13)}$= 8.887 (p=0.000561)				

All the variables with significance $P > 0.05$ were removed through a backward stepwise procedure. In the table also the diagnostics of regression are reported, and namely R^2 and F-test with the corresponding significance (A: *T. vulgaris*; B: *M. piperita*; C: *Z. tenuior*).

500 ml. In each tube, different concentrations of EOs (0, 0.1 and 0.2 % v/v) were added, according to the CCF chosen. The EOs was emulsified (30% of the total weight) with water and Tween 80 (Sigma–Aldrich, USA) by using a Homogenizer mixer (IKA-T25-digital ultra turrax) for 1 minute at 15000 rpm. Each sterilized doogh tube (containing different EO concentrations, according to CCF described) was inoculated with standardized concentration solution in order to obtain an initial *S. aureus* cell concentration of 1×10^5 CFU/ml. A control tube (not added with any antimicrobial substances) was analyzed as *S. aureus* growth control (positive control).

Staphylococcus aureus counts in doogh samples

S. aureus survival in doogh samples was monitored during a refrigerate storage of 24 h and 168 h (7 days). Specifically, 1 mL of doogh was aseptically transferred to 9 ml of 0.9% (w/v) NaCl sterile solution and the resulting suspension was serially diluted in the same diluent and plated onto mannitol salt agar medium (Merck, Germany) incubated at 37 °C for 48 h. Three different tubes for every condition were analyzed for each sampling time.

Statistical analysis

Optimization of EO concentrations was done using RSM (57). The dependent variable (response) was *S. aureus* concentration (log CFU/ml, assessed by plate

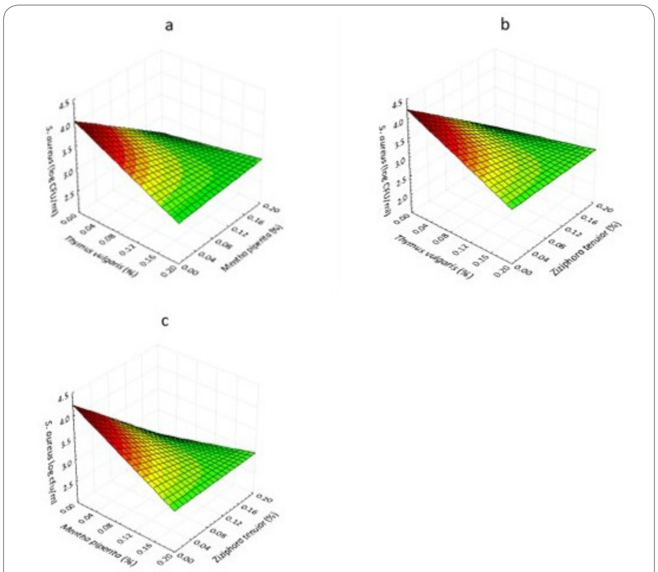

Figure 1. Response surface of *S. aureus* population (log CFU/ml) after 24 h of doogh refrigerated storage. The effect of *T. vulgaris* and *M. piperita*, *T. vulgaris* and *Z. tenuior and M. piperita* and *Z. tenuior* is shown in graph a, b and c, respectively. In each graph, the absent EO was kept constant at the central value of the experimental design (0.1% v/v).

count as described before) at 24h and 168 h of doogh refrigerate storage. Independent variables were the EO concentrations, in particular *T. vulgaris* L. EO (A, [v/v%]), *M. piperita* EO (B, [v/v%]) and *Z. tenuior* L EO (C, [v/v%]). The experimental data were fitted with a second order polynomial model applying the least squares regression to estimate the regression coefficients in the equation. The generalized second-order polynomial model used in the response surface analysis was as follows:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j$$

Where β_0 is an estimated constant, β_i are the fitted regression coeffincients related to the linear term of the covariate x_i (independent variables), β_{ii} are the fitted regression coeffincients related to the quadratic term of the covariate x_i and β_{ij} are the fitted regression coeffincients related to the interaction terms of the covariates x_i x_j. The regression analysis was performed using Statistica 8.1 (StatSoft Italy s.r.l., Vigonza, Italy) and the final model was obtained through a stepwise procedure and including only parameters with $P \leq 0.05$.

Results and Discussion

Polynomial model

The data relative to *S. aureus* counts for each run of the CCF showed in Table 2 were fitted with a second order polynomial equation, in order to evaluate the effects of the presence of the three EOs on the *S. aureus* cell load in doogh. With the aim to simplify the model, a backward stepwise procedure was applied and only the terms characterized by significance higher than 95% were kept in the final model. The data of the linear regression are reported in Table 3. As it is possible to observe, after 24 h and 7 days (168 h) of storage, no quadratic term was significant and only linear and interactive terms were kept in the final model. In particular, a negative sign characterized all the linear terms, while the interactive coefficients were all positive. This final model resulted highly significant, as demonstrated by the Fisher (F) test, which is aimed to evaluate the level of significance (p-values) associated with ANOVA and by the values of the regression coefficients. The presence of the same terms in the final model and the similar coefficients indicated an analogous behavior for *S. aureus* counts at 24 and 168 h of refrigerate storage.

With the aim to visualize this behavior, the response surfaces obtained at 24 and 168 h are reported in Figure 1 and 2. In each graph, the EO not present in the run was kept constant at the central value of the experimental design (0.1% v/v). According to Fig. 1, after 24 h,

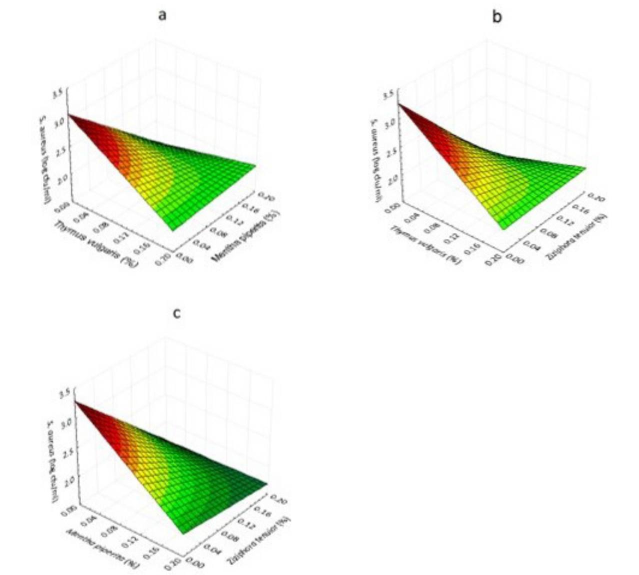

Figure 2. Response surface of *S. aureus* population (log CFU/ml) after 168 h (7 days) of doogh refrigerated storage. The effect of *T. vulgaris* and *M. piperita*, *T. vulgaris* and *Z. tenuior* and *M. piperita* and *Z. tenuior* is shown in graph a, b and c, respectively. In each graph, the absent EO was kept constant at the central value of the experimental design (0.1% v/v).

it is possible to observe a relevant effect of the single EOs on the survival of *S. aureus*. The increase of the concentration of one EOs when the other was not present determined an almost linear decrease of the survival of the pathogen. However, no interactive effect was evidenced at the higher EO concentrations. The samples after 168 h (Figure 2) confirmed this behavior, although the number of survivors was lower. However, also after 7 days no particular interactive or synergistic effect was observed.

The behavior described by these graphs indicated that the increase of each EOs from 0 to 0.2% (v/v) is responsible for *S. aureus* cell reduction. However, if we consider the maximum concentration of one of the EO, no adjunctive inhibition effect was determined by the increase of another EO even to its maximum concentration. Therefore, in these conditions, it is possible to state that we cannot observe any synergistic or additive effects of the three EOs considered in this dairy product. In order to better evidence this fact, Figure 3 represents *S. aureus* cell load (log CFU/ml) after 24 h of storage in relation to *T. vulgaris* and *M. piperita* when *Z. tenujor* was not added (Fig. 3a) or was added at its maximum concentration (Fig. 3b). While in Figure 3a, a relevant decrease of cell load was observed with the progressive increase of each EO concentration, confirming what stated before, Figure 3b, showed that if we consider the model at the maximum concentration of *Z. tenujor*, the effects of concentration variations of *T. vulgaris* and *M. piperita* add scarce or irrelevant effects. The experimental design suggested that the presence of 0.2 % (v/v) of EO, independently of the type, reached the maximum inhibition level.

It is well know that active components of the EOs can interact with the food components, *i.e.* solubilizing in lipids and interacting with proteins (53). Doogh is fermented milk with a high protein and fat content (1) and the EO fractions interacting with these compounds are no more able to reach the cellular target and to exert any antimicrobial action.

The negative public perception of industrially synthesized food antimicrobials has increased the interest in more natural, non-synthesized, antimicrobials as potential alternatives to conventional preservatives to extend shelf life and prevent foodborne pathogens (52). The antibacterial effect of EOs is often related to the antimicrobial effects of specific terpenes. Even if the composition of the EOs from the same plant can be very different, the main components of *Z. tenuior* EO are pulegone, isomenthone, p-menth-3-en-8-ol and 8-hydrohymenthone (58). For *M. piperita*, menthol, menthon, menthofuran, β-caryophyllene and eucalyptol have been found to be the main components (59), while for *T. vulgaris* they are thymol, carvacrol, *p*-cymene and γ-terpinene (60).

The antimicrobial activity of the bioactive compounds has its main target in the cell membrane, disturbing their fluidity and permeability; this perturbation determines several consequences such as membrane potential depletion, loss of cytoplasmic substances and ions up to cell disruption (53).

Several researchers explored the effect of different EOs in many various dairy products. Ghalem and Zouaoui (47) studied the effects of *Lavandula* and *Chamaemelum* species EOs on physicochemical, microbial and organoleptic qualities of yoghurt. They found that these EOs showed remarkable antibacterial activity against bacteria, yeasts and moulds. In another study, the same Authors (48) studied the effect of *Rosmarinus officinalis* EO on microbiological and physico-chemical quality of yoghurt. They demonstrated that yoghurt containing *R. officinalis* EO had a satisfactory hygienic quality due to the absence of any pathogen. Moreover, sensory analysis indicated that the samples added with 0.14g/L of EO improved flavour, taste and texture with respect to the other samples.

Bonyadian and Moshtaghi (61) investigated the effectiveness of five EOs (thyme, tarragon, caraway seed, penney royal and peppermint) on survival of *S. aureus* in Feta cheese and found that thyme and tarragon EOs were the most effective.

Abd-El Fattah et al. (62) investigated lemongrass extract antimicrobial effects on yoghurt and found that 0.1% and 0.3% of lemongrass water extract were effective for inactivating both mould growth and mycotoxin production.

Fazeli et al. (63) studied the antibacterial effect of *Rhus coriaria* and *T. vulgaris* on some foodborne bacteria. They reported that the former Persian spices was

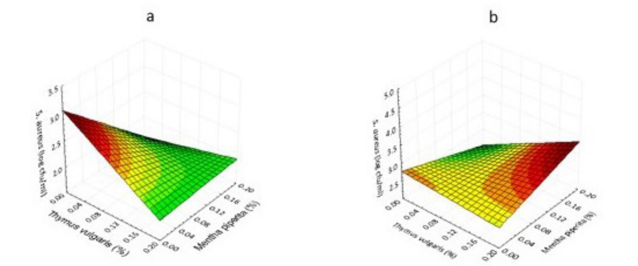

Figure 3. Response surface of *S. aureus* population (log CFU/ml) after 24 h of doogh refrigerated storage in relation to *T. vulgaris* and *M. piperita* when *Z. tenujor* was not added (a) or was added at its maximum concentration (b).

effective against pathogenic bacteria and could be used as natural food additives. Mohamed et al. (49) studied the effects of antimicrobial properties of dill, caraway, coriander, basil and lemon balm EOs on dairy product quality. They observed that caraway and dill EOs had the highest antibacterial effect against the five tested pathogenic bacteria, *i.e. Listeria monocytogenes, Staphylococcus aureus, Bacillus cereus*, *Escherichia coli* O157:H7 and *Salmonella typhimurium*.

Bagamboula et al. (64) investigated the effectiveness of some EOs (thyme and basil) and some of their components (carvacrol, thymol, estragol, linalool and *p*-cimene) against *Shigella sonnei* and *S. flexneri* and demonstrated their effectiveness in reducing their cell numbers below the detection limit at concentration of 1%.

The antimicrobial activity of these EOs has been tested in dairy products, but few data are available on antibacterial influence of *T. vulgaris*, *Z. tenuior* and *M. piperita* EOs in dairy product such as doogh (47, 48, 50, 60). It has been shown that the combination of terpenic compounds either in single EO or their mixtures affects different biochemical processes of the target bacteria, and produces various interactive antibacterial effects (35). For instance, synergism has been observed between the EOs of *Origanum vulgare* and that of *Rosmarinus officinalis* against *Listeria monocytogenes* and *Yersinia enterocolitica* (65).

The effects of natural EOs derived from the *Lamiaceae* plants family on Doogh samples were measured and demonstrated to have an effect on *Staphylococcus aureus*. The results showed that the concentration conditions including *T. vulgaris L*, *M. piperita* and *Z. tenuior* L. EOs influenced the survival of *S. aureus,* which decreased during refrigerate storage. In general, the optimum concentration conditions were obtained at the maximum concentration of one EO, independently from the concentrations of the others. However, slightly lower *S. aureus* survivals were observed at the maximum concentration of *Z. tenuior* EO. In spite of the antimicrobial activity of the tested EOs, further experiments are needed to assess their performance in food matrix and, in particular, in dairy product.

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Conflicts of Interest

The authors declare no conflict of interest.

Author's contribution

A. Abdolshahi, S. Naybandi-Atashi, M. Heydari-Majd, B. Salehi, M. Sharifi-Rad and J. Sharifi-Rad designed the study and carried out the experiments and analyzed the results. B. Salehi, M. Sharifi-Rad, J. Sharifi-Rad, F. Kobarfard, S. A. Ayatollahi, G. Tabanelli, C. Montanari and M. Iriti contributed to write the manuscript. G. Tabanelli contributed to performed second order polynomial model and C. Montanari contributed to performed statistical analysis. M. Iriti, A. Ata, and J. Sharifi-Rad supervised the final version of the manuscript.

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