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 Lamiacea family, such as *Thymus vulgaris* L., *Ziziphus 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 Infectious and Industrial Fungi and Bacteria collection Center in Biotechnology unit of Iranian Research Organization for Science and Technology (Tehran, 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 (H₂SO₄) and 0.5 ml of 1.175% barium chloride dihydrate (BaCl₂•2H₂O). 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 Barj 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.

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

<table>
<thead>
<tr>
<th>Time</th>
<th>Intercept</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>AB</th>
<th>AC</th>
<th>BC</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>5.30</td>
<td>-10.35</td>
<td>-10.55</td>
<td>-12.61</td>
<td>38.50</td>
<td>46.50</td>
<td>34.00</td>
</tr>
<tr>
<td></td>
<td>R²= 0.801</td>
<td>F-test (6,13) = 8.725 (p=0.000604)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>168 h</td>
<td>4.36</td>
<td>-10.71</td>
<td>-10.90</td>
<td>-12.95</td>
<td>39.13</td>
<td>48.13</td>
<td>35.63</td>
</tr>
<tr>
<td></td>
<td>R²= 0.803</td>
<td>F-test (6,13) = 8.887 (p=0.000561)</td>
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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 $1 \times 10^3$ 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 count as described before) at 24 h 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:

\[
Y = \beta_0 + \sum \beta_i x_i + \sum \beta_i x_i^2 + \sum \beta_{ij} x_i x_j
\]

Where $\beta_0$ is an estimated constant, $\beta_i$ are the fitted regression coefficients related to the linear term of the covariate $x_i$ (independent variables), $\beta_{ij}$ are the fitted regression coefficients related to the quadratic term of the covariate $x_i$ and $\beta_{ij}$ are the fitted regression coefficients related to the interaction terms of the covariates $x_i$ and $x_j$. 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,
they are thymol, carvacrol, menthol, and Z. tenuior. The experimen
tal design suggested that the presence of 0.2 % (v/v) of
piperita EOs, independently of the type, reached the maximum
inhibition level.

It is well known that active components of the EOs
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 inte-
rest 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-hydroxymenthone (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 com-
ounds has its main target in the cell membrane, dis-
turbing 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 Cha-
maemelum 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-chemi-
cal 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 ef-
ectiveness 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
equal antimicrobial effects on yoghurt and found that
0.1% and 0.3% of lemongrass water extract were effec-
tive 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 bac-
teria. They reported that the former Persian spices was

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).

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. tenuior 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 Lamiacceae 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*, *M. Piperita* and *Z. tenuior* 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.

References
Trachyspermum ammi and Onco
L., essential oil against
ment of Human Immunodeficiency Virus. International Journal of

34. Salehi B, Kumar NV A, Şener B, Sharifi-Rad M, Kılıç M, Ma
doi: 10.1002/ptr.6103.

25. Sharifi-Rad J, Salehi B, Schnitzler P et al. Susceptibility of

44. Shahbazi Y. The antibacterial effect of Zizia phlorinoides
cidal applications in foods—a review. International Journal of Food
Molecular Sciences 2018; 19(5): 1459.

35. Burt S. Essential oils: their antibacterial properties and poten-
tional applications in foods—a review. International Journal of Food
Microbiology 20014; 94(3): 223-253.

ection to encapsulation. International Journal of Pharmaceutics

37. Patrignani F, Siroli L, Serrazanetti DI, Gardini F, Lanticci R.
innovative strategies based on the use of essential oils and their
ponents to improve safety, shelf-life and quality of minimally processed
s and vegetables. Trends in Food Science and Tech-

38. Seow YX, Yeo CR, Chung HL, Yuk H-G. Plant essential oils as
ctive antimicrobial agents. Critical Reviews in Food Science and

39. Montanari C, Serrazanetti DI, Felis G et al. New insights in ther-
al resistance of staphylococcal strains belonging to the species
phlococcus epidermidis, Staphylococcus lugdunensis and

40. Sharifi-Rad J, Hoseini-Alifentemi SM, Sharifi-Rad M, Setzer
WN. Chemical composition, antifungal and antibacterial activities of

41. Gottardi D, Buvkivic D, Prasad S, Tyagi AK. Beneficial Effects of
ces in Food Preservation and Safety. Frontiers in Microbi-
2016; 7.

42. Shahbazi Y. Ziziphus phlorinoides essential oil and nisin as
potential antimicrobial agents against Escherichia coli O157: H7 in
doogh (Iranian yoghurt drink). Journal of Pathogens 2015; 176024:

43. Karim G, Meshgi MA, Ababil RK, Bokaie S. Antimicrobial Ef-
effect of Mentha spicata and Mentha pulegium Essential Oils in Two Storage Temperatures on the Survival of Debaromyces hansenii in

44. Shahbazi Y. The antibacterial effect of Zizia phlorinoides
cidal activity vs. Staphylococcus aureus. Cell Mol Biol (Noisy le Grand) 2018 | Volume 64 | Issue 8
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50. Asensio CM, Grosso NR, Juliani HR. Quality preservation of
organic cottage cheese using oregano essential oils. LWT-Food Science and Technology 2015; 60(2): 664-671.

51. de Carvalho RJ, de Souza GT, Honório VG et al. Comparative
hibitory effects of Thymus vulgaris L. essential oil against Sta-
phlococcus aureus, Listeria monocytogenes and mesophilic starter
culture in cheese-mimicking models. Food Microbiology 2015;
52: 59-65.

52. Calo JR, Crandall PG, O’Bryan CA, Ricke SC. Essential oils as
antimicrobials in food systems—A review. Food Control 2015; 54:


