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Antimicrobial Effect of *Mentha spicata* and *Mentha pulegium* Essential Oils in Two Storage Temperatures on the Survival of *Debaryomyces hansenii* in Iranian Doogh

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Abstract

Background and Objectives: Doogh is an Iranian preferred dairy drink, especially in warm seasons. Blowing by yeasts is a common occurring spoilage when this product is kept outside the refrigerator in warm temperature. Natural additives such as herbal essential oils, which also induce the desired flavor and color, may solve this problem and retard yeast growth. Spearmint, pennyroyal and some other herbs and their extracts were traditionally used in this product. In the current study, the antimicrobial effect of *Mentha spicata* and *Mentha pulegium* essential oils on the survival of *Debaryomyces hansenii* was evaluated in two different storage temperatures.

Materials and Methods: Iranian Doogh was prepared according to the national standard method with different concentrations of the mentioned essential oils. Then all the samples were inoculated with yeast inocula to achieve a yeast count of $>3\times10^6$ CFUml^{-1}. Viability of *Debaryomyces hansenii* was investigated during the storage time (0-28 days) of Doogh samples at 4°C and 25°C at different intervals. Statistical analysis was performed using the one-way Analysis of Variance and Tukey's post hoc methods. Also α level was considered equal to 0.05.

Results and Conclusion: Different concentrations of *Mentha spicata* and *Mentha pulegium* essential oils had significant effect on the growth of the yeast. Increasing the concentration of these essential oils decreased the logarithm of number of microorganisms. The essential oils of *Mentha spicata* and *Mentha pulegium* in low concentrations have antimicrobial effect on spoilage yeast and can improve the sensory properties of Doogh. Therefore, they can be the best alternative preservatives for hazardous chemical compounds.

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1. Introduction

Doogh as an Iranian fermented dairy drink is traditionally produced from dilution of full fat yogurt after vigorous agitating in special leather bags, called 'Mishk'. Doogh comprises its specific physical, chemical, physicochemical, microbiological and sensory characteristics that are characterized by Codex Alimentarius [1]. Currently there is a strong debate about the safety aspects of chemical preservatives as they are considered responsible for many carcinogenic and teratogenic attributes as well as residual toxicity [2]. These compounds are widely used to extend the shelf life of Doogh and inhibit the yeast's growth. In

the last few years, there has been great interest concerning the inhibitory effect of some plant compounds on food spoilage microorganisms [3,4]. Concerning food, this is due to the increased market demand for the natural preservatives produced from plants or other natural sources [5]. These are Natural-green alternatives for the maintenance or extension of the food products' shelf life. Particular interest has focused on the potential application of plant essential oils [6,7].

Yeasts are widely distributed in nature, and are able to spoil many foods such as yoghurt, Doogh, cheese, vinegar, beverages, juices, fruits, salads, sugars and meat, causing changes in their odor, color, taste and texture [8]. *Debaromycess hansenii* is a non-pathogenic, osmotolerant and oleoginous micro-organism, which is also known to be the agent of spoilage of fermented dairy products [9]. Of the 128 samples of yoghurt examined for the presence of yeasts, 45% exhibited yeast count above 10^3 cells per g; of which, *D. hansenii* was the most frequently isolated species [10].

Among the plant substances, extracts from several plants, traditionally used as flavoring agents, are known to possess antimicrobial properties in different conditions [4,11,12] In particular, essential oils from Lamiaceae family (e.g. oregano, thyme, basil, mint, spearmint (*Mentha spicata)*, pennyroyal (*Mentha pulegium)*, rosemary, siderites and sage) are recognized as effective inhibitors of some important spoilage yeasts, and therefore, could be used as natural food preservatives [3,13].

The shelf life of Doogh is moderately long (approximately one month or lesser); this product should be kept in the refrigerator, but it is often hold in ambient temperature in retail markets. Because of high storage temperature and long storage time, swelling as an undesirable spoilage is caused by some microorganisms such as *D. hansenii* [9]. So this study was designed to evaluate, for the first time, the antimicrobial effect of *Mentha spicata* and *Mentha pulegium* essential oils on the survival of *D. hansenii* in two storage temperatures in Iranian Doogh, and also study the possibility of chemical preservatives replacement by these natural compounds.

2. Materials and Methods

2.1. Plant material

Mentha spicata and *Mentha pulegium* were collected in Tehran province and identified by Iranian Institute of Medicinal Plants.

2.2. Preparation of essential oils

Air-dried aerial part of the plants was subjected to steam distillation for 2 h using Clevenger-type apparatus. The essential oil yield of the air-dried material was analyzed by gas chromatography (Thermoquest 2000, UK). The chromatograph was equipped with DB5 capillary column (30×0.25 mm ID×0.25 μm film thickness) and the data were acquired under the following conditions: initial temperature 50°C, program rate 2.5°C, final temperature 265°C, and injector temperature 25°C. The carrier gas was helium and the split ratio was 120. The essential oil was also analyzed by gas chromatography–mass spectrometry (GC–MS) (Termoques Finningan, UK) and the same capillary column under the analytical conditions mentioned above. The MS was run in the electron ionization mode using the ionization energy of 70 eV.

2.3. Test organism and preparation of inocula

Active culture of *D. hansenii* (DSM 70590) was obtained from the German company of DSMZ (Deutsche Sammlung Von Mikroorganism and Zellkulturen GmbH).The yeast was cultured on Yeast Extract Dextrose Chloramphenicol agar slants, and after 5 days incubation in 25°C, they were stored in the refrigerator and then sub-cultured every two weeks. The inocula were prepared by streaking cells from the storage cultures on the plates of Yeast Extract Dextrose Chloramphenicol agar. After incubation for 5 days at 25°C, a few colonies were transferred to Yeast Extract Dextrose Chloramphenicol broth and incubated at 25°c. The yeasts were grown to late stationary phase and then centrifuged at 11000 ×g for 10 min. the prepared microbial suspension contained more than 10^8 CFU ml^{-1} of *D. hansenii* and was used for inoculation.

2.4. Experimental design

To assess the effect of essential oils on *D. hansenii* in two different temperatures, a laboratory experiment was designed. This design included 6 levels of *Mentha spicata* essential oil (0.05, 0.1, 0.25, 0.5, 1 and 1.5 percent) and 6 levels of *Mentha pulegium* essential oil (0.025, 0.05, 0.1, 0.5, 1 and 1.5 percent) in two storage temperatures (25°C as an ambient temperature and 4°C as refrigeration temperature). The examinations were repeated for growth (viable count) in a food model system (Doogh) at 5 intervals (0, 7, 14, 21 and 28 days) with three replications. Statistical analysis was performed using SPSS ver. 16.0 by one-way Analysis of Variance and Tukey's post hoc methods. Also α level was considered equal to 0.05.

2.5. Preparation of the food model system

A commercial packed 2.5% fat yogurt was mixed with pre-boiled cool water to 60:40 ratio in screw capped flasks under sterile conditions. The yogurt was tested make sure not to contain yeasts. Amount of 0.5% laboratory NaCl (Merck) was added to samples under sterile conditions. Then the essential oils were added to the samples according to the study design. Doogh with no additives was used as control.

2.6. Incubation and storage of Doogh

The screw capped flasks containing 250 ml Doogh with the designated amounts of essential oils were inoculated with test organism in order to contain more

than 3×10^6 CFU ml^{-1}. Ten-fold serial dilutions of the culture were prepared in wv^{-1} peptone water (Merck, KGaA, Darmstadt, Germany). These dilutions were used for confirmation of the number of cells in the cultures by surface plate count on Yeast Extract Dextrose Chloramphenicol agar. The samples were tested initially (day 0) by viable count on Yeast Extract Dextrose Chloramphenicol agar followed by incubation at 25°C and 4°C with viable count on Yeast Extract Dextrose Chloramphenicol agar at specified intervals (7, 14, 21 and 28 days).

2.7. Sensory evaluation

Sensory evaluation of adding essential oils in the concentrations, which are usually used as flavoring agents in Doogh manufacturing (*Mentha spicata* at 0, 0.1, 0.25 and 0.5 percent concentrations and *Mentha pulegium* at 0. 0.05, 0.1 and 0.5 percent concentrations), was done by an acceptance test. The sensory evaluation was performed by a panel of seven judges consisting of trained staff from the Department of Food Hygiene. Each panelist evaluated the samples by rating using a two-point scale (1=acceptable and 0=unacceptable) for various characteristics such as odor, flavor and general evaluation. The variability of acceptance or liking of the samples was analyzed by regression analysis and Mc Nemar test (SPSS 16.0 for windows, SPSS Inc.).

3. Results and Discussion

3.1. Chemical composition of essential oils

Table 1 represents the chemical properties of *Mentha spicata* and *Mentha pulegium* essential oils at 20°C, respectively.

3.2. Organoleptic effect of *Mentha spicata* and *Mentha pulegium* essential oils

Table 2 indicates rating for the acceptability of Doogh samples containing various concentrations of *Mentha spicata* and *Mentha pulegium* essential oils. Despite the strong activity against foodborne pathogens and spoilage microorganisms shown by essential oils, their application is currently limited to their undesirable flavor changes they cause in food products [15], which is not in agreement with our findings.

Table 1. Chemical properties of *Mentha spicata* and *Mentha pulegium* essential oils at 20°C

Essential oils	Specific gravity	Optical rotation	Refractive index	Principal chemical constituent
Mentha spicata	0.929	-55.10	1.487	Carvone (56%)
Mentha pulegium	0.952	+18.12	1.494	Pulegone (37%)

Table 2. The acceptability scores of Doogh samples containing different levels of *Mentha spicata* and *Mentha pulegium* essential oils

	Essential oil (%)	Odor	Flavor	General
Mentha spicata	0.50	1^a	0^a	0^a
	0.25	1^a	0^a	0^a
	0.10	1^a	1^b	1^b
	0.00	1^a	1^b	1^b
Mentha pulegium	0.50	1^a	0^a	0^a
	0.10	1^a	0^a	0^a
	0.05	1^a	1^b	1^b
	0.00	1^a	1^b	1^b

*Numbers followed by the same letter at each column are not significantly different ($p> 0.05$)
1=Acceptable 0=Unacceptable

3.3. Factor effects

Figures 1 and 2 illustrate the growth responses of *D. hansenii* as affected by the essential oils and different temperatures in Doogh during 28 days of storage**.**

3.3.1. Temperature effect

According to Karanika et al. plant extracts have no direct effect on yeast cells [6]. The antimicrobial activity may be attributed to the chelation of metal ions, essential for microbial growth. In foods, not only intrinsic factors such as fat, proteins, water, antioxidants, preservatives, pH, salt and other additives but also extrinsic factors like temperature, package type and properties of microorganisms can affect the sensitivity of microorganisms [14].
Sensitivity of microorganisms to the antimicrobial effect of essential oils increases by decreasing pH, amount of oxygen and increasing food temperature [5,15].

Incubation temperature had a significant effect on growth of yeasts. Increasing the incubation temperature decreased the logarithm of number of yeasts. Logarithm of number of yeasts was significantly different between the two different temperatures (25°C and 4°C) ($p\leq0.01$).

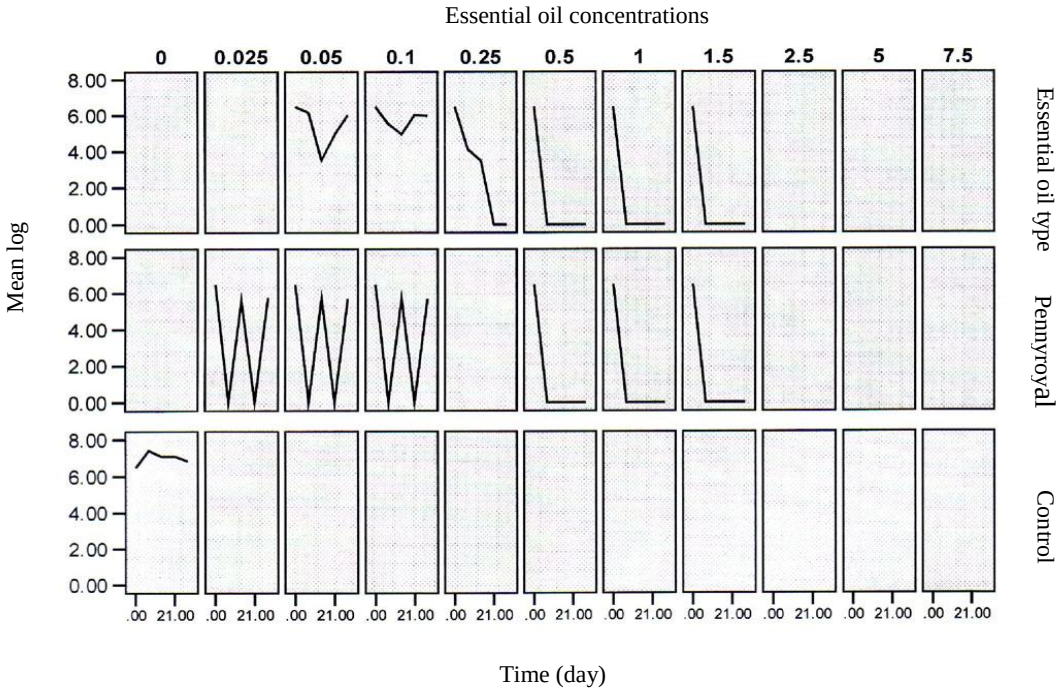

Figure 1. Mean log of the number of yeasts in the samples incubated at 4°C.

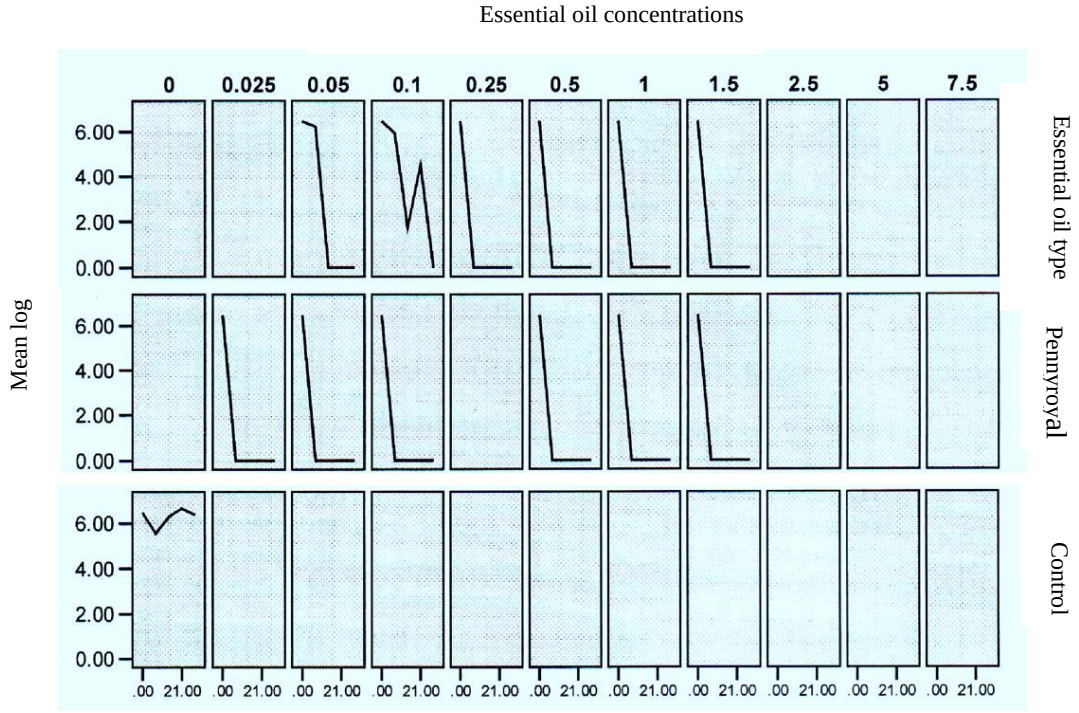

Figure 2. Mean log of the number of yeasts in the samples incubated at 25°C.

Coefficient of correlation between the incubation temperature and the logarithmic number of yeasts was -0.038 and R^2= 0.001.

In the present study, all concentrations of *Mentha spicata* and *Mentha pulegium* essential oils showed a significant antimicrobial effect at 25°C; however, at 4°C, only 0.25, 0.5, 1 and 1.5 percent concentrations of *Mentha spicata* essential oil and 0.5, 1 and 1.5 percent of *Mentha pulegium* essential oil showed this effect. These findings are in agreement with the observations of the above mentioned researchers.

3.3.2 Effect of storage time

Storage time had significant effect on the growth of yeasts. Increasing the storage time decreased the growth rate of yeasts. Coefficient of correlation

between the storage time and logarithmic number of yeasts was -0.109 ($p \leq 0.01$) and R^2=0.01.

3.3.3. Effect of essential oils

a. *Mentha spicata* essential oil

Mentha spicata essential oil had a significant effect on the growth rate of yeasts, and decreased the number of yeasts. Coefficient of correlation between *Mentha spicata* essential oil and the logarithmic number of yeasts was -0.83 ($p \leq 0.01$).

b. *Mentha pulegium* essential oil

Mentha pulegium essential oil had also a significant effect on the growth rate of yeasts and decreased their number. Coefficient of correlation between *Mentha spicata* essential oil and the logarithmic number of yeasts was -0.61 ($p \leq 0.01$).
Also, increasing the incubation temperature decreased the logarithmic number of yeasts. The higher incubation temperatures encourage the fermentation rate, which leads to lowering the pH and increases the sensitivity of microorganisms to the antimicrobial effect of the essential oils. This finding is in agreement with those of other studies [16]; however, some studies did not obtain the same results [17]. This difference may be because of the different types of food models, as well as the type and concentration of essential oils used in different studies.

3.3.4. Effect of essential oil concentration

Different concentrations of *Mentha spicata* and *Mentha pulegium* essential oils had significant effect on the growth of yeasts. Increasing the concentration of these essential oils decreased the logarithmic number of yeasts ($p \leq 0.01$). Coefficient of correlation between the essential oil concentration and the logarithmic number of yeasts was -0.543 and R^2=0.295. It has been reported that dilution of an essential oil will decrease its properties. The antibacterial activity has been attributed to the presence of some active constituents such as carvone and pulegone in the oils [18,19].
In the present work, the logarithmic number of yeasts decreased significantly in all the samples that contained both essential oils and stored at 25°C. *Mentha spicata* essential oil in 0.25, 0.5, 1 and 1.5 percent concentrations and *Mentha pulegium* essential oil in 0.5, 1 and 1.5 percent concentrations significantly decreased the logarithmic number of yeasts at 4°C.

Mentha spicata essential oil in 0.05 and 0.1 percent concentrations and *Mentha pulegium* essential oil in 0.025, 0.05 and 0.1 percent concentrations had no effects on the logarithmic number of yeasts in 4°C. The hydrophobic effect of essential oils increases in low pH and leads to increasing its solubility in plasma membrane lipids [20]. Dissolving of essential oil in the lipid phase of food leads to decreasing its amount in the liquid phase in order to have antimicrobial effect [21].

In the present study increasing the storage time decreased the logarithmic number of the yeast. This is probably due to this fact that our food model (Doogh) becomes more acidified as time passes so more essential oils solve in the lipid layer of yeast cells under more acidified conditions. Decreasing pH increases antimicrobial effect of essential oils [7,22,23].

4. Conclusion

According to the present study results, the essential oils of *Mentha spicata* and *Mentha pulegium* not only have antimicrobial effect against *D. hansenii*, but if used in low concentrations, they can also improve sensory properties of Doogh. The chemical preservatives, used in Doogh to prevent the yeast growth, can be replaced by these essential oils, which have no harm on the human health

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6. Conflict of interest

The authors have not declared any conflict of interest

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