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Antibacterial Effect of *Teucrium polium* Essential oil and *Lactobacillus casei* Probiotic on *Escherichia coli* O157:H7 in Kishk

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Abstract

Background and Objective: In recent years, there has been a rapid development in prevention of bacterial growth and spoilage in foods by the application of preservatives. Essential oils, which are known to be harmless for human, have shown positive health effects in addition to their preservative role. Likewise, probiotics exert improvement in both food product and health level of consumers. With reference to these it is important to do more research on various effects of endemic essential oils on traditional food products. A survey was carried on effect of essential oil and combination of essential oil and probiotics on growth of *Escherchia coli* O157:H.

Material and Methods: Kishk samples treated with *Teucrium polium* essential oil (75 and 150 mg l^{-1}) and *Lactobacillus casei* (10^8-10^9 CFU ml^{-1}) were stored up to 20 days at 4°C and the effect of *Teucrium polium* essential oils and probiotic fermentation on *Escherchia coli* O157:H7 counts were investigated.

Results and Conclusion: *Teucrium polium* essential oils and probiotic fermentation reduced the number of *Escherichia coli* during the cold storage time. The combination of *Teucrium polium* essential oil and probiotics showed stronger effect rather than individually added. Initial count of inoculated *Escherchia coli* was 6.39 log CFU g^{-1}. Essential oil compounds of *Teucrium polium* included Spathulenol, Epizonaren and Bicyclo hept-3-en-2-ol in 18.39, 9.62 and 6.76%, respectively. After 20 days storage at 4°C, the number of *Escherchia coli* in probiotic, combination of probiotic and essential oil (150 mg l^{-1}) and control samples decreased from 6.39 log CFU g^{-1} to 4.95, 4.30 and 5.8 log CFU g^{-1}, respectively. An increase in acidity and decrease in pH were observed for all samples ($p \leq 0.05$). Based on organoleptic tests, the most accepted sample was included 75 ppm of essential oil and probiotic. According to the antibacterial effect of essential oils extracted from plant and probiotic fermentation, it was predictable that they could decline the *Escherchia coli* count especially in fermented dairy products. Also, due to its probiotic nature, Kishk would bring numerous health advantageous for consumer.

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1. Introduction

A vast variety of traditional fermented dried foods products are being produced in many countries. A fresh dried fermented milk product, which is widely produced in the rural regions between the Middle East and the Indian subcontinent, is called Kishk, Kushuk, Keshkeh or Kichk [1]. It consists of low-fat yoghurt or full-fat yoghurt's buttermilk [2]. Kishk properties including specific characteristics had been mentioned in Iran National Standard, though traditional products spoilage are still a major problem. This problem caused by a variety of microorganisms has often been recognized as inconvenient and one of the most important concerns for these products. These contaminations can take place at various stages from production to sale and distribution [3]. Now, to counteract this problem, the manufacturers use chemical preservatives such as benzoic acid, nitrites, and sulphites to prevent the growth of food spoiling microbes [4]. Preservatives in foods are designed to prevent microbial growth and spoilage, but sometimes they can also have negative effect on health. While the effects of food preservatives on the body can vary with age and health status, there have been problems concerning the safety of some of these chemicals, including the possibility of allergies, the formation of carcinogenic components [5]. Compared to chemical preservatives, herbal extracts have demonstrated to be more safe with less health-related problems. They also have shown to be effective in chronic disease prevention and infection control by deterioration of pathogenic microorganisms [6,7]. Also, some of extracted essential oils (EO) are aromatic and an important source of new chemical substances that improve organoleptic, physicochemical properties, nutritional values and shelf life of food products. *Teucrium* (*T.*) *polium* is one of these herbs that provide mentioned objectives. *T. polium* locally named Kalpooreh has been known as an important traditional medicinal plant in Khuzestan, South West of Iran which is a member of Lamiaceae family [8]. Antimicrobial, anti-diabetic, anti-inflammatory, antispasmodic, analgesic and antioxidant effects are some of the valuable effects of this plant [9].

In addition to herbal essential oils, the use of probiotic bacteria in food products can improve nutritional value of these products. Probiotic foods, which have drawn attention for decades are used for fortification of great abundance of foods, including fermented milk based products, fruit juices, cereal bars, infant formulas and Kishk [10]. Probiotics products are enhancing health agents because they have been shown to down-regulate inflammation in the gastrointestinal tract, and also having immune functional effects [11]. Based on several studies some strains are chosen due to their especial health effects on human, but it is vital that the safety aspects must be considered carefully.

Escherichia (*E.*) *coli* O157:H7 is a hazard for public health and has been concerned in many epidemics of hemorrhagic colitis and hemolytic uremic syndrome result in fatalities [12]. In the USA, about 75,000 cases of *E. coli* infection was estimated in 2011. The mortality rate of the disease, the possibility of large-scale outbreaks from food supplies and the absence of an effective treatment have impelled thorough research on the pathogenesis and detection of *E. coli* [13].

Screening for transferrable antibiotic resistance genes and unwanted metabolic activities together with collecting evidence are some techniques for demonstration of history of using safe food additives [14].

In this study, we investigated the effects of *Kalpooreh* (*T. polium*) EOs on *E. coli* in probiotic Kishk for possible decrease *E. coli* count and to increase the safety of the product. There are few studies on the combination of endemic preservative along with traditional Iranian food products. This study pursued this purpose in order to decrease chemical preservatives and as well as the amount of salt and show a way to use endemic plants as natural preservatives, at least, in the Iranian food products.

2. Materials and Methods

2.1. EO extraction

In April, 2015, the aerial parts of *T. polium* were gathered from South of Iran (Kerman province). The botanical identification of the sample was conducted by the herbarium located in the faculty of pharmacy, University of Tabriz, Iran. The special parts of plants were separated, cleaned and dried under shade at room temperature for one week. Dried plants were finely ground with a blade-carbide grinding (IKA-WERK Type: A: 10, USA) afterwards. Then, prepared powder was kept in tight containers used as a protective tool against U.V light.

In the next process, essential oil was extracted via distillation by water for 3 h using a Clevenger

-unit steam distillation (Clevenger-type apparatus, European Pharmacopia, France) accomplished on 100 g of *T. polium* for two hours in order to produce the desired EO [15]. Anhydrous sodium sulfate was used to remove the last traces of water in the obtained EO. Finally, extracted oil was stored in dark glass bottles at 4°C until it was used.

2.2. GC-MS Analysis of *T. polium* EO

The essential oil was analyzed by gas chromatography, according to the method of Ehsani and Mahmoudi [16]. In order to analyzing EO, the chromatograph (Agilent 6890; Agilent Technologies, USA) was equipped with an HP-5MS capillary column (30 m×0.25 mm i.d. ×0.25 µm film thickness) and the data were taken under the conditions as follows: Initial temperature = 50°C, temperature ramp = 5˚C min^{-1}, to 300˚C as final temperature (three min holding time) and injector temperature = 290˚C. Helium was the carrier gas and the split ratio was 0.8 ml min^{-1}.

2.3. Bacterial strains preparation

Lyophilized *E. coli* was attained from the culture collection of the Department of Microbiology, Faculty of Veterinary Medicine, Tabriz, Iran. Both sub cultivation and preparation of the inoculate were according to Mahmoudi et al.[4] Lyophilized bacteria were transferred to BHI broth medium (Merck company, Germany) and incubated for 18 h at 37°C and re-cultured for at least 2 times consecutively. For calculation of the required amount of bacteria (10^5 CFU ml^{-1}) for inoculation, spectrophotometer (manufactured by Pharmacia Company, England) reading was done at 600 nm, and bacteria were cultured through surface method and counted [17].

2.4. Probiotic starter culture preparation

A commercially lyophilized *Lactobacillus* (*L.*) *casei* culture ATCC 3939 was obtained from the Iranian Organization of Industrial Research (Tehran, Iran). Sub cultivation and preparation of the probiotic bacteria were conducted based on the method of national Phillip et al. [18] method.

2.5. Inoculation and preparation of Kishk samples

Kishk were bought from a local store, taken to the laboratory and stored in refrigerator. Then, collected Kishk were divided into six samples. The samples comprised: A (control, without EO and probiotic), B (Kishk containing probiotic, without EO), C (containing 75 mg l^{-1} EO), D (with 150 mg l^{-1} EO), E (containing 75 mg l^{-1} EO and probiotic) and F (with 150 mg l^{-1} EO and probiotic). The samples were stored in refrigerator at 4°C, for 20 days. Samples were prepared and transported by the means of non-conductor boxes containing ice packs. In the normal procedure of *E. coli* O157:H7 enumeration pre-enrichment and enrichment steps are used to detect low levels of *E. coli* O157:H7 in food samples. An aliquot of 25 g food sample is added to 225 ml BPW and incubated at 37°C for 24 h. Following incubation, an aliquot (1 ml) is dispensed into 10 ml EC broth (reduced bile salts supplemented with novobiocin) and incubated at 37°C for 24 h.

For *E. coli* O157:H7 a loopful of the EC broth is streaked onto Sorbitol MacConkey (SMAC) Agar (supplemented with cefixime and potassium-tellurite) and incubated at 37°C for 24 h .Dilution stage took place by adding phosphate buffered saline (PBS, Merck, Germany). A portion of 25 g of each samples was blended with 225 ml nutrient broth (Merck, Germany) for two min, using a Stomacher lab blender (A33, PRC) and incubated at 37°C for 24 h. Afterwards, 1 ml of nutrient broth culture was mixed up with 9 ml of MacConkey broth (Merck, Germany) and then incubated at 37°C for 24 h. One loop of each tube was streaked on MacConkey agar (Merck, Germany). Such colonies were confirmed as *E. coli* using standard biochemical tests (i.e., Indole, Methyl red, Voges-Proskauer and Citrate utilization tests) [19]. Primary population of inoculated *E. coli* was 6.39 log CFU g^{-1}.

2.6. Physicochemical analyses

Physicochemical properties were including pH, acidity and ash. These properties determined according to other scientific studies [20,21].

2.7. Sensory evaluation

The sensory effects of adding of *T. polium* EO and probiotic bacteria to Kishk were evaluated using an acceptance test. The sensory evaluation was performed by a panel of five trained evaluator consisting of the scientific staff of the Department of Food Hygiene, Faculty of Veteri- nary Medicine, University of Tabriz, experienced in the sensory analysis of food. Water was provided for mouth washing between samples. Each panelist evaluated the samples by rating using a nine-point scale, where 9 = like extremely, 8 = like very much, 7 = like moderately, 6 = like slightly, 5 = neither like nor dislike, 4 = dislike slightly, 3 = dislike moderately, 2 = dislike very much and 1 = dislike extremely, for various characteristics such as color (appearance) , odor and flavor.

2.8. Statistical analysis

The statistical analysis was conducted with SPSS 18.0 software (Chicago, IL, USA). Results were reported as a mean±standard deviation and were subjected to one-way ANOVA to establish whether the differences in experimental results were significant or not. $p \leq 0.05$ was assumed as significant level and all the tests were conducted in triplicate.

3. Results and Discussion

3.1. Chemical analysis of the *T. polium* EO

In the present study the EO was extracted by the hydro distillation of the dried aerial parts of *T. polium* and yield of the EO was 0.5% (w w^{-1}). GC and GC-MS analysis enabled the identification of a total of 64 constituents. Among these some components were the major ingredients available in the *T. polium* EO which are shown in Table 1, presented according to their retention time and the relative concentrations of the identified volatile components (%).

Table 1. The composition of *T. polium* (Kalpooreh) EO[§§] determined by GC-MS.

Compound Name	RT[§] (min)	Percentage % (v w^{-1})
Spathulenol, 1h-Cycloprop	18.08	18.39
Epizonaren	19.37	9.62
Bicyclo (3.1.1) Hept-3-en-2-ol	8.60	6.76
D-Germacrene	15.65	6.33
Trans-Caryophyllene	14.26	6.17
P-Mentha-1,5-dien-8-ol	9.02	3.76
7-Epi-Alpha-Selinene	19.46	3.49
Bicyclo[3.1.1]Hept-2-ene-2-Methanol	9.61	3.34
2-Naphthalenemethanol	18.64	3.02
Bicyclogermacrene	15.94	2.91
Caryophyllene	18.44	2.05
Caryophyllene Oxide	17.13	1.80
Delta.-Cadinene	16.46	1.75
Caryophyllenol-ii	19.60	1.74
Junipene	18.95	1.70
Trans-.Beta.-Farnesene	14.82	1.60
Bicyclo[3.1.1]Hept-3-en-2-one	9.96	1.57
1H-Cycloprop[e]azulene	16.13	1.46
Beta. Bourbonene	13.40	1.42
Veridiflorol	18.31	1.42
Alpha.-Caryophyllene	14.90	1.41
Acetic acid	11.50	1.28
Aromadendrene	19.71	1.26
Trans-(+)-Carveol	10.11	1.24
2h-Cycloprop[e]azulene	18.76	1.17
Naphthalene	19.02	1.16
Salvial-4(14)-en-1-one	18.18	1.14
Butyl Hydroxy Toluene	16.68	1.06
Beta.-Cadinene	16.39	1.05
Total		91.07

[§] RT: retention time (min)

[§§] EO: essential oil

The main components found were spathulenol (18.39%), Epizonaren (9.62%), Bicyclo heptenol (6.76), D-Germacrene (6.33%) and, trans-Caryophyllene (6.17%). The EO of *Teucrium* species has been studied in Iran and in the world. In a study on *T. polium*, EO compounds, Spathulenol (15.06%), Beta-Pinene (11.02), Beta-Myrcene (10.05), Germacrene B (10.11%), Germacrene D (8.15%), Bicyclogermacrene (8.25%) and Linalool (4.02) have been shown as the major components of the oil [22]. A study on the EO obtained from seven *Teucrium* species (*T. arduini L., T. botrys L., T. chamaedrys L., T. flavum L., T. montanum L., T. polium L., T. scordium L.*) identified More than 60 compounds. The main constituents of *T. arduini and T. chamaedrys* oils were β-caryophyllene (24.5% and 26.9%) and germacrene D (21.9%; 22.8%), respectively. The oil of *T. botrys* contained β-caryophyllene (20.4%), α-humulene (13.9%) and (E)-β-farnesene (17.7%), while *T. flavum* oil contained α-pinene (17.5%), β-pinene (11.5) and β-bisabolene (35.0%). *T. scordium* oil's main component were reported α-pinene (17.7%) and β-pinene (10.0%), while *T. montanum* oil contained α-pinene (12.4%), germacrene D (15.0%) and β-eudesmol (10.1%), β-pinene (19.8%), and germacrene D (11.9%) [23]. Forty-five constituents were detected in the EO obtained from the fruit of *T. polium*. The major components identified were α-pinene (18.2%), elemol (14.5%), β-pinene (10.1%), cubenol (10.0%), and limonene (5.0%) [24]. Based on the results of GC-MS analysis of *T. polium* EO in our study, Spathulenol, Epizonaren, Bicyclo (3.1.1) hept-3-en-2-ol, D-Germacrene, Trans-Caryo-phyllene were the major components and also these components have been reported as the major compounds of EO obtained from *Teucrium* species in other scientific research. However, a wide range of variety in percentage and even in the main components in each study is obvious. It is possible to say that the difference in the quality or quantity of the composition of volatile oils may be due to genetic, differing chemo type, drying conditions, mode and part of plant for distillation and or extraction and geographic or climatic factors.

3.2. Physicochemical properties

Titrable acidity (TA) and pH

The results showed the effect of storage and treatments on the pH values of various Kishk samples are presented in Figure 1. As we can see, a consistent decrease in pH along the storage was noted, based on results the initial pH values for the different Kishk types ranged from 4.59 to 3.92. The results are in line with the findings of Golestan [25] et al. and Castro [26] et al. They reported a decrease in pH of some fermented dairy products during storage. The observed pH range of 4.59 to 3.92 in our Kishk samples is still within the range reported by earlier research [25-27]. The reason for the decrease in pH is a function of acidity that was increased during the storage period due to the conversion of lactose to lactic acid. Taken together, it appears that the composition of starter culture, fermentation temperature, storage duration, contamination, etc., could influence the overall level of acidity and pH of stored fermentative dairy products [28]. Shahdadi et al. studied the effects of various essential oils (mint, *Mentha spicata*; bee balm, *Mentha longifolia*; eucalyptus, *Eucalyptus camaldulensis* and ziziphora *Ziziphora tenuior* L) on some characteristics and probiotic activities in yoghurt during storage time. A gradual increase in acidity (from 0.75 to 1.2) was observed whereas a decline in pH (from 4.3 to 3.77) was noted in all the samples [29].

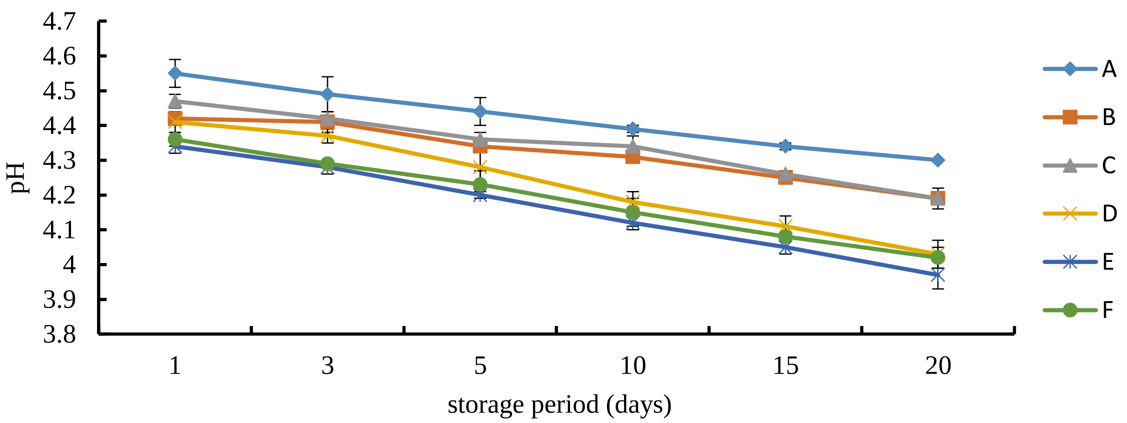

Figure 1. Changes in pH value of Kishk containing essential oil (EO) during the storage

A: Kishk control, B: Kishk+ probiotic, C: Kishk+ EO (75 mg l^{-1}), D: Kishk+ EO (150 mg l^{-1}), E: Kishk + EO (75 mg l^{-1}) + Probiotic, F: Kishk + EO (150 mg l^{-1}) + Probiotic

Since TA affects the shelf life and the organoleptic acceptance of fermented dairy products, it is an important factor. Based on results in Figure 2, TA was increased during the cold storage of Kishk. However, at early days, there was no significant change in the titratable acidity by additives, but after some day storage we can see a significant difference ($p \le 0.05$) in titratable acidity of control and treated samples especially in essential oil and probiotic containing treatments. Kishk samples containing 75 mg l^{-1} of the EO and *L. casei* had the highest TA when fresh and it increased up to the end of storage, it can be concluded that the EO had a specified effect on the starter culture and *L. casei*. These results were in agreement with other researchers [21]. As they reported that the TA increased gradually during storage period.

Ash

Ash contents of various Kishk samples which are shown in Table 2 showing non-significant increase throughout the storage period. This might be due to the metabolic activities by lactose-fermenting bacteria. This increase in ash content could be a result of incomplete use of minerals by fermenting organisms during their metabolism [30]. In 2016 a study was done on the impact of *Spirulina platensis* on Physicochemical Properties of Probiotic UF Feta Cheese. The result showed that adding the *Spirulina platensis* biomass significantly increased ($p \le 0.05$) the amount of iron, protein and hardness of probiotic feta cheese during the refrigerated storage at 4°C [31]. A study about the effect of some essential oils on shelf life of concentrated yoghurt (labneh) show non-significant increase in the total solid, fat to dray matter and protein content of the samples, either when fresh or during the storage. Increased total solid, fat to dray matter could be due to moisture loss [32].

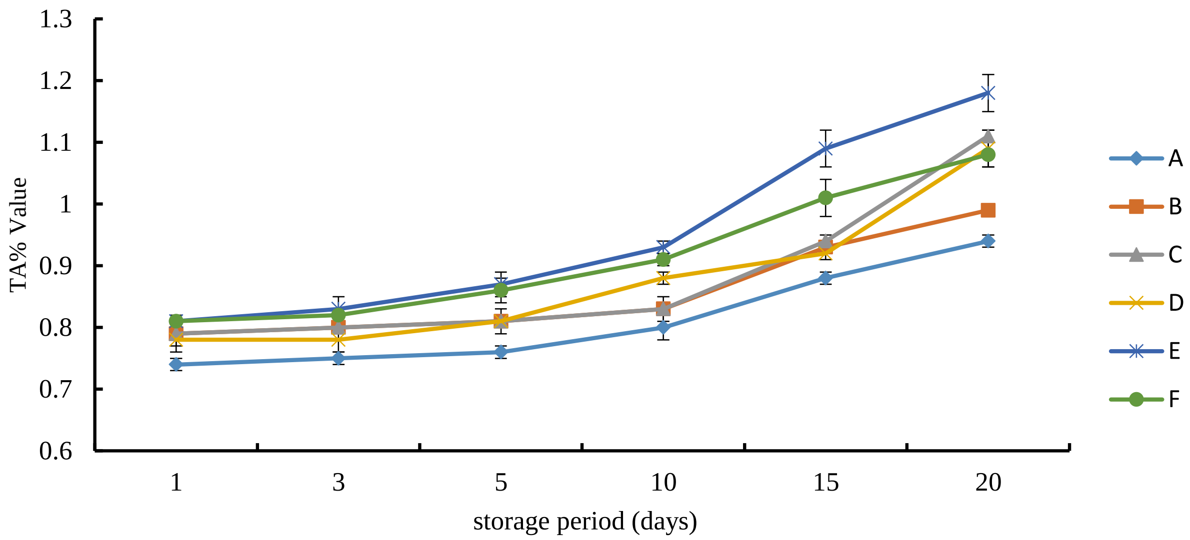

Figure 2. Changes in TA (%) of Kishk samples containing essential oil (EO) during the storage

A: Kishk control, B: Kishk+ probiotic, C: Kishk+ EO (75 mg l^{-1}), D: Kishk+ EO (150 mg l^{-1}), E: Kishk + EO (75 mg l^{-1}) + Probiotic, F: Kishk + EO (150 mg l^{-1}) + Probiotic

Table 2. Changes in ash (%) of Kishk samples during the storage

Kishk samples	Storage times (days)					
	1	3	5	10	15	20
A	1.98±0.03^b	2.01±0.04^b	2.07±0.06^b	2.14±0.03^b	2.18±0.02^b	2.21±0.02^b
B	1.99±0.07^b	2.06±0.05^b	2.09±0.04^b	2.12±0.03^b	2.15±0.03^b	2.18±0.04^b
C	1.98±0.03^b	2.03±0.05^b	2.10±0.06^b	2.14±0.05^b	2.18±0.04^b	2.24±0.06^b
D	1.92±0.03^a	1.95±0.04^a	2.01±0.04^a	2.12±0.02^a	2.18±0.02^a	2.21±0.02^a
E	1.91±0.04^a	1.95±0.03^a	2.01±0.03^a	2.09±0.01^a	2.15±0.01^a	2.19±0.01^a
F	1.90±0.02^a	1.95±0.01^a	2.03±0.03^a	2.11±0.01^a	2.12±0.01^a	2.23±0.04^a

A: Kishk control, B: Kishk+ probiotic, C: Kishk+ essential oil (75 mg l^{-1}), D: Kishk+ essential oil (150 mg l^{-1}), E: Kishk + essential oil (75 mg l^{-1}) + Probiotic, F: Kishk + essential oil (150 mg l^{-1}) + Probiotic

3.3. Survival of *L. casei*

According to Figure 3, addition of the *T. polium* EO in different concentrations (75 and 150 mg l^{-1}) to Kishk samples significantly increased *L. casei* count during the storage time on 20 days ($p \leq 0.05$). Combining the effect of medicinal plant extract and probiotics may be a new approach due to their complementary antimicrobial effects and practically no side effects. However it was observed that different strains have different sensitivity to essential oils. The synergistic effect of the essential oil and probiotics will be necessarily higher than using alone. Among gram positive bacteria, Lactic acid bacteria (LAB) are often known as the most resistance species against antimicrobial effects of EO [33]. There are several studies showing more stability of LAB against EO in comparison with Pathogens. EO can even maximize the growth of LAB by inhibiting the growth of other microorganisms [4,22,34,35]. Kishk contain probiotic bacteria without the EO had the lowest total viable count of *L. casei*. (6.8 CFU g^{-1}). Effects of the *T. polium* EO on *L. casei* viability were significantly different in 75 and 150 mg l^{-1} concentrations ($p \leq 0.05$). Result of enumeration of the *L. casei* showed that Kishk samples containing 75 mg l^{-1} EO had the highest total viable count (7.34 CFU g^{-1}) of probiotic bacteria, when compared with Kishk containing 150 mg l^{-1} of the EO (6.97 CFU g^{-1}) and the Kishk containing just probiotics ($p \leq 0.05$). It was reported that different concentrations of herbal essential oils can influence the survival and activity of starter bacteria and LAB in fermentative dairy products [21,36]. Survival of probiotics and their activity is dependent to EO concentration. Our results showed that bio-Kishk containing 75 mg l^{-1} of had the highest total viable count. These results are in agreement with those obtained by Simsek et al. and mahmoodi et al. which showed that addition of herbal essences such as mint, thyme and garlic, improve the survival and activity of probiotics [21,23]. Among Gram-positive bacteria, LAB are often known as the most resistant species against antimicrobial agents of herbs [33]. In a study which was designed by Sarabi et al., essential oil of *Mentha piperita* and *Ziziphora clinopodioides* were added to inoculated milk in different concentration (0, 25, 40, 70, 100, 130 µg l^{-1}). All treatments were incubated at 42°C until medium acidity reached 0.6%. Viability of *Lactobacillus acidophilus* during the storage of bio yoghurt at 4°C at different time intervals was investigated. The results showed that the number of starter culture significantly ($p \leq 0.05$) decreased after 7 days of storage [37]. According to FAO, a standard probiotic product must contain a minimum of 10^6-10^7 CFU g^{-1} live and active probiotic microorganisms at the moment of consumption [38]. In this study, durability of *L. casei* at the end of storage period of control Kishk and Kishk containing *T. polium* EO (especially in 75 mg l^{-1}) was sufficient to exert beneficial health effects.

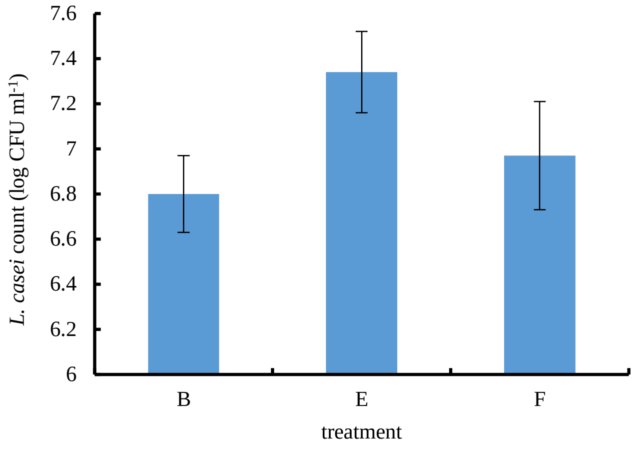

Figure 3. Survival of *Lactobacillus casei* after 20 day storage at 4°C (log CFU ml^{-1})

Sample B: probliotic Kishk with no added EO,
Sample E: probiotic Kishk with 75 mg l^{-1} EO,
Sample F: probiotic Kishk with 150 mg l^{-1} EO.
Error bars represent the standard deviation**.**

3.4. Survival of *E. coli*

Survival of *E. coli* (log CFU g^{-1}) in control and probiotic Kishk samples over 20 days at 4°C have shown in Table 3. The results in this Table indicate that, *T. polium* EO reduced the *E. coli* counts during and after the 20-day storage at 4°C. (6.27 to 4.95 log CFU g^{-1} for Kishk included 75 mg l^{-1} EO and 6.11 to 4.54 log CFU g^{-1} for Kishk contain 150 mg l^{-1} of EO) Also results showed that probiotic inoculation in addition to *T. polium* EO leads to declination more in *E. coli* counts during the storage time. (6.14 to 4.84 log CFU g^{-1} for sample with probiotic and 75 mg l^{-1} of EO and also 5.91 log CFU g^{-1} to 4.30 for Kishk with probiotic and 150 mg l^{-1} EO) Therefore, the combined antimicrobial effects of probiotic and EO in this study were more effective than that was used in individual one. Reviewing literature showed that some EOs have special antibacterial special effects which can be applied as preservatives, flavoring agents with even health promoting benefits [39]. Mahmoudi *et al.* revealed synergistic inhibitory effect of *T. polium* EO and *L. casei*, as a probiotic, on *Salmonella typhimurium* population during 28 days of yoghurt storage. They found that the combination of 40 mg l^{-1} *T. polium* EO along with *L. casei* (10^8-10^9 CFU ml^{-1}) decreased the required inhibitory concentration of *T. polium* EO. [21]. Singh *et al*. declared that the total amount of *Anis* EO (1000 mg l^{-1}) and oleoresin has greater influence on controlling the reduction of microorganisms causing spoilage in yoghurt [28]. Dehkordi *et al.* also found that some factors like virulence, number of serogroups and antibiotic resistance genes showed a high level of resistance to more than one antibiotic in the *E. coli* strains isolated from yoghurt, doogh and Kishk. Their results showed that O157 and non-O157 STEC strains were superior in traditional dairy samples [40]. In another study, Osaili *et al.* reported that the enumeration of *E. coli* inoculated in plain yoghurt constantly declined from 7.69 log CFU g^{-1} to 1.47 log CFU g^{-1} after 8 days of storage at 4°C. The results of the population of the pathogenic bacteria in yoghurt and kefir samples indicated a reduction at various levels during the storage time [5,41]. Our study presented the evidence fact that *T. polium* EOs and probiotic fermentation lead to a significant reduction in *E. coli* total count at individual and complex status. However, antimicrobial effects of complex one (essential oil along with probiotic) was better. Some researchers also showed the synergistic effects of EOs extracted from plant material and probiotic fermentation against pathogenic microorganisms [4,22].

Organoleptic properties

The organoleptic properties of the different Kishk samples and the average results over 20 d3.ays storage are presented in Figure 4. There were considerable and significant differences ($p \leq 0.05$) in the flavor of samples treated by 150 mg l^{-1} of EO as compared with the untreated control. The Bio Kishk (containing probiotic) by adding 75 mg l^{-1} EO, when fresh and after 20 days of storage, was preferred compared to another treated Kishk with probiotic. It can be concluded that 75 mg l^{-1} of *T. polium* EO can be used in order to increase the shelf life of Kishk with acceptable flavor and good appearance without any signs of spoilage organisms. Acceptance of Kishk with lower concentration of EO can be correlated to tendency of normal and usual taste of food compare with new taste of those same foods. In a scientific study on oregano essential oil on traditional Argentinean cheese no differences in the organoleptic characteristics of semi-hard cheeses flavored with oregano essential oil (200 mg l^{-1}) and homemade cheeses flavored with oregano leaves have been found [42]. In a study on effect of *Mentha spicata* L. and *Mentha aquatic* L. essential oils on the Kishk, samples containing *M. aquatic* EO at 1500 and 2500 mg l^{-1} were the most preferred samples [25]. Sadeghi et al. studied effects of *Mentha pulegium* essential oil at concentrations of 0.03, 0.015, and 0.0075% in Iranian white cheese during 60 days of storage. They found that although 0.03% concentration of mentha oil had the most strong antibacterial effects, but samples with 0.015% essential oil had significantly higher organoleptic properties score compared to other samples ($p \leq 0.05$) [43].

Table 3. Effects of *T. polium* essential oil (EO) on *E. coli* counts in Kishk samples during storage at 4°C

Time (day) Treatment	1	3	5	10	15	20
A (log CFU g^{-1})	6.39±0.01 ^{a}	6.30±0.08 ^{a}	6.17±0.04 ^{a}	6.04±0.01 ^{a}	5.93±0.01 ^{a}	5.80±0.01 ^{a}
C (log CFU g^{-1})	6.27±0.05 ^{a}	6.07±0.02 ^{b}	5.96±0.03 ^{a}	5.78±0.02 ^{b}	5.50±0.02^{b}	4.95±0.05 ^{b}
D (log CFU g^{-1})	6.11±0.07 ^{b}	5.95±0.09 ^{b}	5.82±0.01 ^{b}	5.68±0.08 ^{c}	5.43±0.05 ^{b}	4.54±0.09 ^{c}
B (log CFU g^{-1})	6.27±0.02^{a}	6.17±0.05 ^{a}	6.00±0.06 ^{a}	5.95±0.05 ^{b}	5.85±0.03 ^{a}	5.65±0.04 ^{c}
E (log CFU g^{-1})	6.14±0.04 ^{b}	6.00±0.03 ^{b}	5.92±0.09 ^{a}	5.74±0.03 ^{b}	5.36±0.08 ^{b}	4.84±0.03 ^{c}
F (log CFU g^{-1})	5.91±0.09 ^{c}	5.84±0.02^{c}	5.72±0.07 ^{c}	5.60±0.05 ^{b}	5.20±0.03 ^{c}	4.30±0.01 ^{d}

A: Kishk control, B: Kishk+probiotic, C: Kishk+ EO (75 mg l^{-1}), D: Kishk+ EO (150 mg l^{-1}), E: Kishk + EO (75 mg l^{-1}) + Probiotic, F: Kishk + EO (150 mg l^{-1}) + Probiotic
Means ± SD in the each column with different letters are significantly different ($p \leq 0.05$).

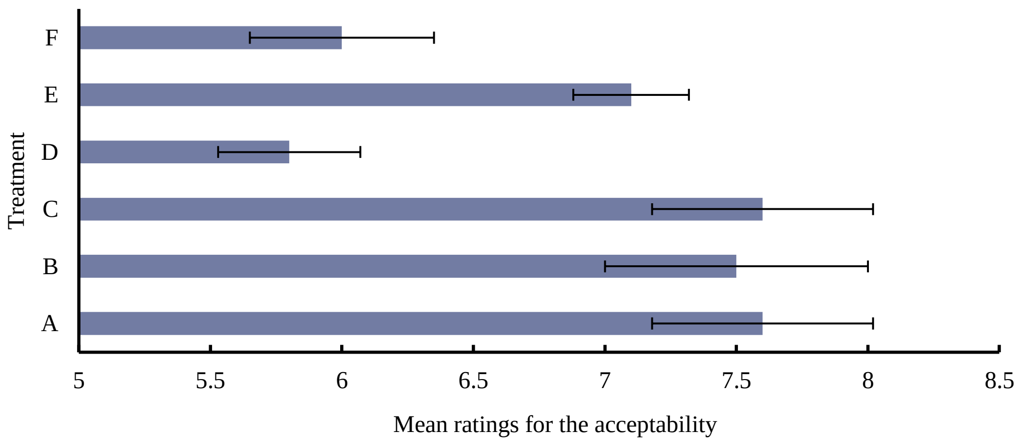

Figure 4. Organoleptic properties of Kishk samples formulated with *T. polium* essential oil (EO) and *L. casei*

A: Kishk control , B: Kishk+probiotic, C: Kishk+ EO (75 mg l^{-1}), D: Kishk+ EO (150 mg l^{-1}), E: Kishk + EO (75 mg l^{-1}) + Probiotic, F: Kishk + EO (150 mg l^{-1}) + Probiotic

However each essential oil has its own effect on organoleptic properties of final product but scientific studies mainly report that essential oils in specific concentration not only has positive effect on shelf life of foods,but they can also improve organoleptic properties.

4. Conclusion

This study confirm that *T. polium* EOs and probiotic fermentation reduced the population of *E. coli* significantly during the storage time; Complex effect of both *T. polium* Eos and probiotics was stronger than using them individually application. According to antimicrobial effects of EOs, extracted from plant material and probiotic fermentation, it is recommended to use this method for reducing the *E. coli* growth and population in food systems; especially in low pH food such as Kishk, a lactic acid fermented dairy product. Due to its probiotic nature, Kishk would bring several health advantageous for consumer.

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6. Conflict of Interest

The authors claim that there is no conflict of interest.

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اثر ضد میکروبی اسانس روغنی *تئوکرایوم پولیوم* و زیست‌یار (Probiotic) لاکتوباسیلوس کازئی بر *اشرشیا کلی* O157:H7 در کشک

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چکیده

سابقه و هدف: در سال‌های اخیر، استفاده از نگهدارنده برای جلوگیری از رشد و فساد میکروبی در مواد غذایی رشد سریعی داشته است. اسانس‌های روغنی که به عنوان موادی بی‌خطر برای انسان شناخته می‌شوند، در کنار اثرات مثبت سلامتی‌بخش خود نقش نگهدارندگی نیز دارند. همچنین، زیست‌یارها موجب بهبود فرآورده غذایی و سطح سلامتی مصرف‌کننده‌ها می‌شوند. از این رو، انجام تحقیقات بیشتر درخصوص اثرات گوناگون اسانس‌های روغنی گیاهان بومی بر فراورده‌های سنتی مهم می‌باشد. در این بررسی اثر اسانس روغنی و تلفیق اسانس و زیست‌یار بر رشد *اشرشیا کلی* O157:H مطالعه شده است.

مواد و روش‌ها: نمونه‌های کشک تیمار شده با اسانس روغنی *تئوکرایوم پولیوم* (کلپوره) در دو غلظت $75mg\ l^{-1}$ و ۱۵۰ و *لاکتوباسیلوس کازئی* ($CFU\ ml^{-1}$ ۱۰۹-۱۰۸) به مدت ۲۰روز در درجه حرارت ۴ درجه سلسیوس نگهداری شدند و اثرات اسانس روغنی *تئوکرایوم پولیوم* و تخمیر زیست‌یار بر تعداد *اشرشیا کلی* O157:H7 مورد مطالعه قرار گرفت.

یافته‌ها و نتیجه‌گیری: اسانس *تئوکرایوم پولیوم* ونیز تخمیر زیست‌یار تعداد باکتری های *اشرشیا کلی* را در مدت نگهداری در انبار سرد کاهش دادند. استفاده توام اسانس *Teucrium polium* و زیست‌یار اثر ضد میکروبی قوی‌تری را در مقایسه با استفاده هر یک به تنهایی نشان داد. شمارش اولیه باکتری‌های *اشرشیا کلی* تلقیح شده$log\ CFU\ g^{-1}$ ۶/۳۹ بود. ترکیبات تشکیل‌دهنده اسانس *تئوکرایوم پولیوم* شامل اسپاتولنول، اپیزونارن و بایسیکلو هپت -۳- ان-۲- ال به ترتیب ۱۸/۳۹، ۹/۶۲ و ۶/۷۶ درصد بود. پس از ۲۰ روز نگهداری در درجه حرارت ۴ درجه سلسیوس، تعداد *اشرشیا کلی* در تیمارهای حاوی زیست‌یار، و توام زیست‌یار و اسانس ($150mg\ l^{-1}$) و نمونه شاهد به ترتیب به ۶/۳۹، ۴/۹۵، ۴/۳۰و ۵/۸ $log\ CFU\ g^{-1}$ کاهش یافت. افزایش تدریجی در میزان اسیدیته و کاهش درpH در همه نمونه ها مشاهده گردید ($p\leq0.05$). با در نظر گرفتن اثرات ارگانولپتیکی اسانس روغنی، بهترین تیمار برای ترکیب ppm ۷۵ اسانس و زیست‌یار به دست آمد. با توجه به اثرات ضد میکروبی اسانس‌های روغنی گیاهان و نیز تخمیر زیست‌یارها، کاهش تعداد *اشرشیا کلی* به خصوص در فرآورده‌های تخمیری شیر قابل پیش‌بینی بود. علاوه بر این، کشک به علت ماهیت پروبیوتیکی یا زیست‌یاری خود اثرات سلامتی بخش بی‌شماری برای مصرف‌کننده به همراه دارد.

تعارض منافع: نویسندگان اعلام می‌کنندکه هیچ تعارض منافعی وجود ندارد.