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Effect of *Ziziphora clinopodioides* Essential Oil Stress on Viability of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* Microencapsulated with Alginate-Chitosan and Physicochemical and Sensory Properties of Probiotic Yoghurt

Nafiseh Alighazi, Negin Noori*, Hassan Gandomi, Afshin Akhondzadeh Basti

Department of Food Hygiene, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

Abstract

BACKGROUND: The probiotics must be alive in sufficient numbers and one of the main stress factors that probiotic strains should tolerate is food preservatives, like herbal essential oils (EOs). To provide a balance between sensory acceptability and antimicrobial efficacy, the use of sub-lethal concentrations of EOs in combination with other preservation methods has been proposed.

OBJECTIVES: The aim of this study was to evaluate the effect of sub-lethal level of *Ziziphora clinopodioides* essential oil (ZEO) stress on viability of microencapsulated *Lactobacillus acidophilus,* and *Bifidobacterium bifidum*, and examine physicochemical and sensory properties of probiotic yoghurt during 28 days of storage. Moreover, the survival of probiotics was evaluated in gastrointestinal conditions.

METHODS: The sub-lethal and lethal levels of ZEO were determined for *Lactobacillus acidophilus* and *Bifidobacterium bifidum*. Both probiotics (10^9 CFU/mL) were exposed to sub-lethal dose of ZEO on MRS broth for about 2 h and then microencapsulated with alginate-chitosan. First, viability of encapsulated probiotics was estimated in simulated gastrointestinal conditions. After preparation of yoghurt, enumeration of free and encapsulated probiotics in yoghurt was done. Finally, physicochemical and sensory properties of probiotic yoghurt were measured.

RESULTS: According to the GC-MS, Thymol (41.70%), alpha-terpineol (7.31%) and carvacrol (5.39%) were the most commonly detected components in the ZEO. The lethal doses of ZEO for *L. acidophilus* and *B. bifidum* probiotic bacteria were 1750 and 1500 ppm, respectively. Encapsulation and exposure of probiotics to sub-lethal dose of ZEO increased significantly the survival of probiotics in both gastrointestinal conditions and during 28 days of yoghurt storage ($P<0.05$). Furthermore, encapsulation and exposure of probiotics to sub-lethal dose of ZEO did not significantly change the pH of yoghurt samples ($P>0.05$). On the other hand, syneresis was not significantly different in all samples ($P>0.05$). The group exposed to ZEO obtained the lowest score for flavor. However, significant differences were observed between the exposed and other groups in the term of flavor, texture and overall acceptability ($P<0.05$).

CONCLUSIONS: Exposure to sublethal concentration of ZEO could be used as a prebiotic in probiotic yoghurt containing probiotics so as to improve the survival and viability of microcapsulated probiotics and enhance some of the physicochemical and sensory properties.

KEYWORDS: *Bifidobacterium bifidum*, Encapsulation, *Lactobacillus acidophilus*, Probiotic yoghurt, *Ziziphora clinopodioides* essential oil,

Correspondence

Negin Noori, **:** Department of Food Hygiene, Faculty of Veterinary Medicine,University of Tehran. Qareeb Street, Azadi AvenueTel: +98 (021) 61117067, Fax: +98 (021) 66933222, Email: nnoori@ut.ac.ir

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Introduction

Yoghurt is a fermented dairy product popular among people all over the world. It is a complete source of minerals such as calcium, proteins, fats and some kinds of useful microorganisms such as *Streptococcus therm-ophilus* (*S. thermophilus*) and *Lactobacillus bulgaricus* (*L. bulgaricus*). In recent years, scientists have tried to increase the organoleptic and health properties of yoghurt using different methods (Fernandez and Marette, 2017). Incorporation of probiotic bacteria into yoghurt is one of the most effective ways to greatly facilitate the improvement of the health charac-teristics of this product (Senadeera *et al.*, 2018; Fenster *et al.*, 2013).

Probiotics are defined as living microrganisms, when ingested in adequate quantities in yoghurt, beneficially influence the health of the host by improving the composition of intestinal microflora. Moreover, probiotics may play a beneficial role in several medical conditions, including lactose intolerance, cancer, allergies, hepatic disease, *Helicobacter pylori* infections, urinary tract infections, hyperlipidemia and assimilation of cholesterol (Tasi *et al.*, 2019). Using beneficial probiotic bacteria such as *Lactobacillus acidophilus* (*L. acidophilus*) and *Bifidobacterium bifidum* (*B. bifidum*) is a suitable way to increase nutritional, physicochemical, sensory and rheological properties of yoghurt. *L. acidophilus* and *B. bifidum* are normal human intestinal flora with considerable probiotic properties. They are recognized for their applications in dairy products, particularly yoghurt (Evivie *et al.*, 2017).

The results of some recent investigations on probiotic products have shown that probiotic organisms cannot resist in fermented dairy products, and also in gastrointestinal conditions. Furthermore, various probiotic lactobacilli and bifidobacteria have shown a decline in their viability during products shelf life (Millette *et al.*, 2013; Pitino *et al.*, 2012). Thus, it is essential to increase the growth, viability and survival of *L. acidophilus* and *B. bifidum* in probiotic dairy products. Using prebiotics is one of the best ways to enhance the growth, viability and survival of probiotic bacteria. Prebiotics are food ingredients that induce the growth or activity of beneficial probiotic microorganisms (Tasi *et al.*, 2019; Evivie *et al.*, 2017).

The genus *Ziziphora* belongs to the *Lamiaceae* family and consists of four species: *Z. clinopodioides* Lam, *Z. persica* Bunge, *Z. capitata* L., and *Z. tenuior* L. This plant is widely distributed in different parts of Iran. Fresh leaves and stems were commonly used as sedative, carminative, appetitive, antiseptic, stomach tonic, wound-healing material, bron-cho expectorant, and antiseptic. ZEO is rich in useful antioxidants such as 1, 8-cineole, pule-gone, carvacrol, thymol, limonene and cymene. Moreover, the air-dried aerial parts of the plant were traditionally used in culinary as spice in different foods such as meat, cheese and yoghurt to enhance their flavor and aroma (Shahbazi, 2017; Smejkal *et al.*, 2016). Furthermore, ZEO contains a large variety of minerals, amino acids, lipids, vitamins and even carbohydrates. Thus, it can be used as prebiotic to improve the growth and survival of probiotic bacteria. Several documented data revealed that inoculation of ZEO into different types of probiotic products caused significant increase in growth, viability and survival of probiotic bacteria, especially *L. acidophilus* and *B. bifidum* (Mahmoudi *et al.*, 2017; Ziaolhagh and Jalali, 2017).

Another way to increase the survival of probiotic bacteria in food matrix and also gastrointestinal condition is microencapsulation. Microencapsulation is a novel method through which a target compound is covered by a thin layer of polymeric material. In this technique, a variety of functional agents, including flavors, EOs, enzymes, and microorganisms, are the

most considered target substances. Microencapsulation technique has been investigated for enhancing the viability of probiotic microorganisms in both dairy products and gastrointestinal tract (Sarao and Arora, 2017; Samedi. and Charles, 2019).

There is limited literature regarding the app-lication of sublethal dose of natural EOs and also microencapsulation to improve survival of probiotic bacteria in yoghurt. Thus, the present research was done to assess the effect of ZEO and microencapsulation with alginate-chitosan on viability of *L. acidophilus*, and *B. bifidum* bacteria, and sensory and physicochemical properties of probiotic yoghurt.

Materials and Methods

Preparation of Inoculum

B. bifidum (Bb-12) and *L. acidophilus* (La-5) were obtained from Chr. Hansen Company (Hørsholm, Denmark). Probiotics were cultured in de Man Rogosa Sharpe (MRS, Merck, Germany) broth at 37ºC for 24 h. Then, activated culture was diluted in fresh media (1%) and incubated at 37ºC. This procedure was performed three times in a week and the slant cultures on Brain Heart Infusion (BHI, Merck, Germany) were stored at 4ºC (Noori *et al.*, 2017).

Plant Materials and Essential Oil Preparation

Fresh aerial parts of *Z. clinopodioides* were collected from Tehran province during full flowering period in March–July 2019. The plants were identified as *Z. clinopodioides* Lam. by a botanical taxonomist. Voucher specimens of plants were deposited in the botany herbarium of the Research Center of Natural Resources of Tehran, Iran. Aerial parts were carefully washed with distilled water and then air-dried indoor in a shady place at room temperature for 12 days (water content approached 75% of plant fresh weight). After that, The ZEO was obtained according to the previously method published by the European Pharmacopoeia (Counsil of Europe, 1997). The dried-sample (100 gr) was grounded and homogenized in distilled water with a ratio of 1:5 and submitted to hydro-distillation for 3.5 h using a Clevenger-type apparatus. The oil over water was recovered, dried with anhydrous sodium sulfate, sealed in brown glass bottle and stored at dark in refrigerator conditions until analysis.

Gas Chromatography–mass Spectrometry (GC–MS) Analysis of EO

Analytical gas chromatography was conducted on a Thermo Quest Finningan apparatus fitted with HP-5MS 5% phenyl methylsiloxane capillary column (30 m length × 0.25 mm i.d. and 0.25 µm film thickness). Helium (purity: 99.99%; flow rate 1.2 mL/min and split ratio 1:20) was used as a carrier gas. Column temperature was initially set at 50°C, then gradually increased to 265°C at a rate of 2.5°C/min and finally fixed at 280°C. The EO analysis was also run on Thermo Quest Finningan coupled to mass spectrometer with the same analytical conditions as indicated above. The MS was run in the electron ionization mode, using the ionization energy of 70 eV (Azizkhani *et al.* 2013).

Detection of Lethal and Sub-lethal Concentrations of ZEO on Probiotics

The *La-5* (10^9 colony forming units (CFU)/mL) and *Bb-12* (10^9 CFU/mL) were inoculated on tubes contained 5 mL MRS broth media with different concentrations of ZEO (0, 1500, 1750, 2000 and 2500 ppm). The *La-5* and *Bb-12* were incubated at 37°C for 2 hr. The culture of probiotics was carried out on time Zero (prior to incubation) and after 2 h incubation. Serial dilutions of cultures were prepared. The selected dilutions were superficially cultured on plates contained the MRS bile agar for the *La-5* and MRS agar with 0.05% L-cysteine and 0.3% sodium propionate for the *Bb-12*. The colonies were then enumerated per each milliliter of media. The lethal dose was determined as a concentration in which at least 2 log decrease

of probiotic survival found and previous concentrations were determined as sublethal doses (De Souza *et al.*, 2016).

Probiotic and EO Exposure

The *La-5* (10^9 CFU/mL) and *Bb-12* (10^9 CFU/mL) were exposed to sublethal dose of ZEO on MRS broth for about 2 hr. The tubes were then centrifuged (4000 rpm) for about 10 min at 4°C and following washing for 3 times with PBS and centrifugation, the OD of bacterial solution was adjusted to 1 (Nasab *et al.*, 2018).

Bacterial Encapsulation

The extrusion of encapsulation was done according to the method described by Krasaekoopt *et al.* (2004) (Krasaekoopt *et al.*, 2004) as follows: Sodium alginate 4% (w/v) solution (Sigma-Aldrich, Steinheim, Germany) was prepared and sterilized at 121ºC for 15 min. For the preparation of chitosan solution, low-molecular-weight chitosan (≥75% deac-etylation, Sigma- Aldrich) (0.4 gr) was mixed with 90 mL of acidified distilled water (acidified with 0.4 ml glacial acetic acid). The pH was adjusted to 5.7–6 by adding 1 mol/L NaOH. Subsequently, chitosan solution was filtered within Whatman qualitative filter paper No. 4 and its volume was adjusted to 100 mL before being autoclaved at 121ºC for 15 min. For encapsulation, 5 mL of bacterial culture (1.5×10^9 CFU/mL) was suspended in 10 mL of sodium alginate solution. The suspensions were extruded dropwise via a 0.11 mm needle into a sterile hardening solution (0.1 mol/L CaCl2). After 30 min of gelification in CaCl2, the beads were washed with distilled water, immersed in 100 mL of chitosan solution and then were shaken on an orbital shaker at 100 rpm for 40 min. The chitosan-coated beads were washed with distilled water and used on the same day.

Viability of Encapsulated Probiotics in Simulated Gastrointestinal Conditions

The simulated gastric juice (SGJ) comprised of 9 g/L NaCl (Merck, Darmstadt, Germany) and 3 g/L pepsin (Sigma-Aldrich) was adjusted to pH 2 with HCl. The aliquots of 0.1 g of encapsulated bacteria or 0.1 mL of free cell suspensions were blended with 5 mL SGJ and incubated for 30 and 60 min at 37ºC with persistent agitation at 50 rpm. To prepare the simulated intestinal juice (SIJ), a solution of 3 g/L ox gall (Merck, Germany) and 1 g/L pancreatin (Sigma-Aldrich) were provided. Sterilization of the solutions was done at 121ºC for 15 min. The aliquots of 0.1 gr of beads or 0.1 mL of cell suspensions were integrated to 5 mL SIJ and incubated for 60 min at 37ºC with the same persistent agitation as for SGJ. After incubation, the beads were disintegrated in sodium citrate solution and the cell count was done using the surface plate technique. The measurement of survival percentage of free and encapsulated *La-5* and *Bb-12* was done with the following equation (Sultana *et al.*, 2000):

Survival (%) = (number of viable cells after exposure to gastrointestinal conditions/number of viable cells before exposure to gastro-intestinal conditions) × 100.

Yoghurt Preparation

Low fat milk (1.5%) was obtained from the Kalleh Company (Amol, Iran). Dry matter of milk was adjusted to 12 to 15% using skimmed milk powder. The mix was then pasteurized at 85ºC for 30 min and cooled up to 45ºC. Afterward, yoghurt starter, 10^9 CFU/g of free and encapsulated of *La-5* and *Bb-12* bacteria, exposed and unexposed to EO were added to the mixture and incubated up to pH 4.6. Then, the prepared yoghurt samples were cooled up to 4ºC and then stored for about 28 days. All analysis was performed on days 1, 7, 14, 21 and 28 (Bertrand- Harb *et al.*, 2003).

Enumeration of Free and Encapsulated Probiotics in Yoghurt

For the enumeration of free and encapsulated probiotics in samples, theyoghurts (10 gr) were re-suspended in 90 ml 0.1% (w/v) peptone water and 90 ml sodium citrate solution, respect-

ively. Serial dilutions were prepared (up to 10^{-6}) and 1 mL of selected dilutions of the *La-5* and *Bb-12* were cultured on MRS bile agar and MRS agar with 0.05% L-cysteine and 0.3% sodium propionate, respectively using pour plate technique. The *La-5* and *Bb-12* were incubated in aerobic and anaerobic conditions at 37ºC for 48 h, respectively (Van de Casteele *et al.*, 2006; Vinderola and Reinheimer, 1999).

Measurement of Syneresis of Yoghurt Samples

The yoghurt samples (20 gr) were subjected to centrifugation at 4ºC (4000 rpm for about 20 min). The supernatant was evacuated and weighted. The syneresis percent was measured according to the relation of the supernatant weight to the primary yoghurt weight (Sahan *et al.*, 2008).

pH Measurement

The pH of yoghurts was determined during the storage time. Each yoghurt sample (1 g) was mixed with distilled water (1:1), and pH was measured using a pH meter (Jenway, UK), calibrated routinely with fresh pH 4.0 and 7.0 standard buffers (Zainoldin and Baba, 2009).

Sensory Evaluation

The taste, texture, appearance and overall acceptance of yoghurt samples were analyzed during the storage time. Sensory analysis was performed using 7 panelists familiar with the sensory properties of yoghurt using 5-point hedonic scale (Hamedi *et al.*, 2014).

Statistical Analysis

All tests were performed in triplicate. The collected data were analyzed using SPSS for Windows Version 21.0 (SPSS Inc., Chicago, IL, USA) and the results were expressed as mean ± standard deviation (SD). The differences in parameters among groups were evaluated using One-Way Analysis of Variance (ANOVA). Duncan was performed as post-hoc multiple comparison test. Statistical significance was set at $P<0.05$.

Results

Chemical Components of ZEO

Table 1 represents the chemical components of ZEO. A total of 50 chemical components (98.15%) were detected in the ZEO. The most commonly detected chemical components in the ZEO were thymol (41.70%), alpha-terpineol (7.31%), carvacrol (5.39%), linalool (4.12%) and gamma-terpinene (4.10%).

Table 1. Chemical components of *Z. clinopodioides* EO.

No	Chemical component	Retention time (min)	Frequency (%)
1	alpha-Thujene	6.194	0.25
2	alpha-Pinene	6.42	1.38
3	Camphene	0.907	0.44
4	(-)-beta-Pinene	7.909	0.14
5	beta-Myrcene	8.494	0.63
6	l-Phellandrene	8.992	0.11
7	alpha-Terpinene	9.496	0.85
8	Cymene	9.865	3.02

No	Chemical component	Retention time (min)	Frequency (%)
9	1,8-Cineole	10.102	2.56
10	trans-beta-Ocimene	10.872	0.3
11	gamma-Terpinene	11.349	4.1
12	cis-sabinene hydrate	11.678	0.32
13	Cis-Linalool Oxide	11.904	0.48
14	Trans-Linalool Oxide	12.592	0.58
15	Linalool	13.311	4.12
16	Camphor	15.047	0.95
17	Borneol L	16.156	2.65
18	4-Terpineol	16.665	1.24
19	Alpha-Terpineol	17.445	7.31
20	6-Octen-1-ol, 3,7-dimethyl-	19.597	0.37
21	Carvacrol Methyl Ether	19.997	0.59
22	Z-Citral	20.203	0.12
23	Linalyl Acetate	20.634	0.33
24	Geraniol	21.132	2.47
25	2,6-Octadienal, 3,7-dimethyl-	21.718	0.16
26	(-)-Bornyl acetate	22.293	0.38
27	Thymol	23.233	41.70
28	Carvacrol	23.469	5.39
29	(+)-2-Carene	25.112	3.45
30	Eugenol	25.364	0.12
31	Piperitenone Oxide	25.662	0.27
32	Copaene	25.939	0.09
33	Geranyl acetate	26.396	1.6
34	trans-Caryophyllene	27.598	2.04
35	Germacrene-D	27.942	0.12
36	(+)-Aromadendrene	28.291	0.14
37	γ-Muurolene	29.677	0.44
38	Germacrene D	29.811	0.70
39	γ-Muurolene	30.288	0.43
40	γ-Cadinene	30.946	0.59

No	Chemical component	Retention time (min)	Frequency (%)
41	delta-Cadinene	31.259	0.87
42	Cis-Alpha-Bisabolene	31.896	0.87
43	Valencene	32.142	0.16
44	cis-Geraniol	32.466	0.17
45	Nerolidol	32.62	1.28
46	(+) spathulenol	33.036	0.11
47	Caryophyllene oxide	33.149	0.88
48	Geranyl propionate	33.76	0.08
49	alpha-Cadinol	34.946	0.61
50	Caryophyllenol-II	35.85	0.19
	Total		98.15

Determination of Lethal and Sub-lethal Doses of EO on Probiotic Bacteria

Figure 1 and 2 represent the survival of *Bb-12* and *La-5* exposed to different concentrations of ZEO, respectively. The lethal doses of ZEO for *La-5* and *Bb-12* were obtained 1750 and 1500 ppm, respectively.

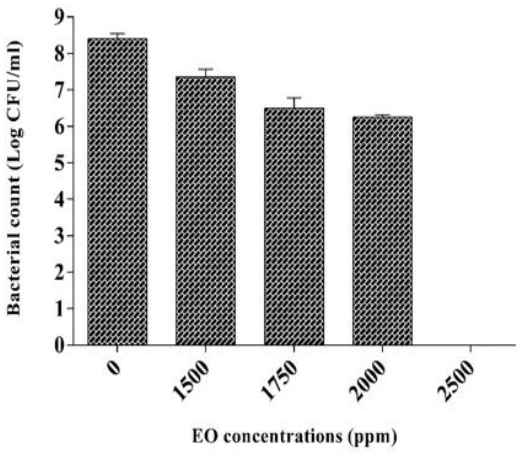

Figure 1. Survival of *L. acidophilus* facing different concentrations of *Z. clinopodioies* EO.

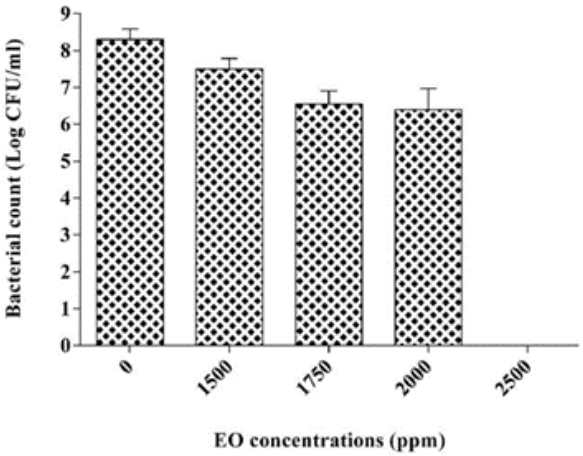

Figure 2. Survival of *B. bifidum* facing different concentrations of *Z. clinopodioies* EO.

Effect of ZEO on Yield of Encapsulation

The numbers of live encapsulated probiotics were measured before and after exposure to ZEO. The *La*-5 exposure to EO (47%) had the highest encapsulation yield which was signi-fi-cantly higher than unexposed *La-5* (32%) (P<0.05). Encapsulation yields in the exposed

Table 2. Effect of *Z. clinopodioides* EO and encapsulation on viability of *L. acidophilus* in simulated gastro-intestinal conditions.

L. acidophilus groups	Survival of bacteria (%)		
	30 min in stomach condition	60 min in stomach condition	60 min in intestine condition
Control	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]
Simple exposed	86.7±0.02[b]	74.4±0.08[b]	62.3±0.08[b]
Capsulated exposed	97.5±0.7[d]	94.4±0.08[d]	83.4±0.06[d]
Simple unexposed	80.7±0.06[a]	78.5±0.1[a]	59±0.2[a]
Capsulated unexposed	96.3±0.06[c]	82.2±0.12[c]	67.7±0.11[c]

*Dissimilar letters in each column show significant difference about $P<0.05$.

and unexposed *Bb-12* were 43% and 30%, respectively.

Effect of ZEO on the Survival of Probiotics in Simulated Gastrointestinal Conditions

Table 2 represents the effect of ZEO and encapsulation on viability of *La-5* in simulated gastrointestinal conditions. The mean survival percent of *La-5* decreased in all tested groups during the storage time in gastrointestinal conditions. Survival of encapsulated *La-5* was significantly higher than non-encapsulated bacteria ($P<0.05$). Additionally, exposure to ZEO significantly increased the survival of *La-5* compared to non-exposed group ($P<0.05$). The encapsulated exposed *La-5* had the highest survival in the first stomach condition (30 min) (97.5% ± 0.7), the second stomach condition (60 min) (94.4%±0.08) and intestine condition (60 min) (83.4%±0.06) ($P<0.05$). Simple unexposed *La-5* had the lowest survival rate in all tested gastrointestinal conditions ($P<0.05$).

Table 3 represents the effect of ZEO and encapsulation on the viability of *Bb-12* in simulated gastrointestinal conditions. The mean survival percent of *Bb-12* decreased in all tested groups during the storage time in gastrointestinal conditions. The survival of encapsulated *Bb-12* was significantly higher than non-encapsulated bacteria ($P<0.05$). Additionally, *Bb-12* exposed to ZEO showed significantly increased survival compared to non-exposed group ($P<0.05$). The encapsulated exposed *Bb-12* had the highest survival in the first stomach condition (30 min) (91.3% ± 0.07) and intestine condition (60 min) (75.7%±0.04) ($P<0.05$). The encapsulated unexposed *Bb-12* had the highest survival rate in the second stomach condition (60 min) (88.1%±0.26). Simple unexposed *Bb-12* bacteria had the lowest survival rate in all tested gastrointestinal conditions ($P<0.05$).

Table 3. Effect of *Z. clinopodioides* EO and encapsulation on viability of *B. bifidum* in simulated gastro-intestinal conditions.

B. bifidum groups	Survival of bacteria (%)		
	30 min in stomach	60 min in stomach	60 min in stomach
Control	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]
Simple exposed	82.2±0.07[b]	78.4±0.07[b]	68.8±0.11[b]
Capsulated exposed	91.3±0.07[d]	85.1±0.06[d]	75.7±0.04[d]

B. bifidum groups	Survival of bacteria (%)		
Simple unexposed	81.7±0.09[a]	73.8±0.04[a]	61.3±0.07[a]
Capsulated unexposed	89.4±0.17[c]	88.1±0.26[c]	72.1±0.39[c]

*Dissimilar letters in each column show significant difference about $P<0.05$.

Effect of ZEO on the Survival of Probiotics in Yoghurt Model in Simulated Gastrointestinal Conditions

Table 4 represents the effect of ZEO and encapsulation on the viability of *La-5* in yoghurt model in simulated gastrointestinal conditions. The survival percent of encapsulated *La-5* in yoghurt was significantly higher than non-encapsulated bacteria ($P<0.05$). Moreover, *La-5* exposed to the ZEO significantly increased the survival of probiotics in comparison with non-exposed group ($P<0.05$).The encapsulated exposed *La-5* bacteria had the highest survival percent in the first stomach condition (30 min) (88.4%±0.1) and intestine condition (60 min) (72.4%±0.2) ($P<0.05$). The encapsulated unexposed *La-5* had the highest survival rate in the second stomach condition (60 min) (82.2%±0.3). Simple unexposed *La-5* had the lowest survival percent in all tested gastrointestinal conditions ($P<0.05$).

Table 5 represents the effect of ZEO and encapsulation on viability of *Bb-12* in yoghurt model in simulated gastrointestinal conditions. The survival percent of encapsulated *Bb-12* in yoghurt was significantly higher than non-encapsulated bacteria ($P<0.05$). Furthermore, *Bb-12* exposed to the ZEO significantly increased the survival of probiotics in comparison with non-exposed groups ($P<0.05$). The encapsulated exposed *Bb-12* had the highest survival percent in the first stomach condition (30 min) (74.2%±0.5), the second stomach condition (60 min) (85.5%±0.3) and intestine condition (60 min) (63.4%±0.2) ($P<0.05$). Simple unexposed *Bb-12* had the lowest survival percent in the second stomach condition and intestine condition, while encapsulated unexposed *Bb-12* had the lowest survival percent in the first stomach condition ($P<0.05$).

Table 4. Effect of *Z. clinopodioides* EO and encapsulation on viability of *L. acidophilus* in yoghurt model simulated gastrointestinal conditions.

L. acidophilus groups	Survival of bacteria (%)		
	30 min in stomach condition	60 min in stomach condition	60 min in intestine condition
Control	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]
Simple exposed	68.6±0.1[a]	74.7±0.1[b]	51.3±0.1[b]
Capsulated exposed	88.4±0.1[c]	81.9±0.1[c]	72.4±0.2[d]
Simple unexposed	67.8±0. 9[a]	70.8±0.2[a]	48.3±0.4[a]
Capsulated unexposed	82.4±0 [b]	82.2±0.3[c]	67.7±0.2[c]

*Dissimilar letters in each column show significant difference about $P<0.05$.

Table 5. Effect of *Z. clinopodioides* EO and encapsulation on viability of *B. bifidum* in yoghurt model simulated gastro-intestinal conditions.

B. bifidum groups	Survival of bacteria (%)		
	30 min in stomach condition	60 min in stomach condition	60 min in intestine condition
Control	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]
Simple exposed	69.4±0.3[a]	72.1±0.4[b]	50.1±0.6[b]
Capsulated exposed	74.2±0.5[b]	85.5±0.3[d]	63.4±0.2[d]
Simple unexposed	69.6±0. 3[a]	69.2±0.5[a]	48.2±0.5[a]
Capsulated unexposed	68.8±0/4 [a]	83.6±0.4[c]	57.5±0.3[c]

*Dissimilar letters in each column shows significant difference about $P<0.05$.

Enumeration of probiotic bacteria in yoghurt samples during the storage time

Table 6 represents the count of *La-5* in yoghurt samples during the storage time. The *La-5* counts decreased in all studied groups. The encapsulation had no significant effect on the survival of *La-5* in yoghurt samples during the storage time ($P>0.05$). However, exposure of *La-5* to the ZEO improved significantly the viability during the storage time ($P<0.05$). Yoghurt samples treated with encapsulated exposed *La-5* had the highest numbers of bacteria in days 1 (8.61±0.2 log CFU/g), 7 (7.99±0.0 log CFU/g), 14 (7.75±0.3 log CFU/g), and 21 (7.45±0.1 log CFU/g) of storage time. The yoghurt samples treated with simple exposed *La-5* had the highest numbers of bacteria in day 28 (7.30±0.3 log CFU/g) of storage time.

Table 7 represents the count of *Bb-12* in yoghurt samples during the storage time. The encapsulation had no significant effect on the numbers of *Bb-12* in yoghurt samples ($P>0.05$). However, *Bb-12* exposed to the ZEO significantly increased the viability during the storage time ($P<0.05$). The yoghurt samples treated with encapsulated exposed *Bb-12* had the highest numbers of probiotic in days 1 (8.25±0.1log CFU/g), 7 (8.09±0.4 log CFU/g), 14 (7.69±0.1 log CFU/g), 21 (7.46±0.1 log CFU/g) and 28 (7.08±0.3 log CFU/g) of storage time.

pH Condition

Table 8 represents the pH of different treatments of yoghurt samples during the storage time. The pH of all studied yoghurt samples decreased during the storage time. No statistically significant difference was observed among the pH contents of yoghurt samples treated with encapsulated and free probiotics ($P>0.05$). Additionally, exposure to the ZEO did not cause significant changes in the pH content of yoghurt samples ($P>0.05$).

Syneresis

Table 9 represents the percent of syneresis of different treatments of yoghurt samples during the storage time. Additionally, exposure to ZEO and encapsulation did not cause significant changes in the syneresis of yoghurt samples ($P>0.05$). However, yoghurt samples treated with encapsulated probiotics unexposed to the ZEO had the highest syneresis (21.93%±0.04) in day 28, while control group had the lowest syneresis (18.28%±0.18).

Table 6. Count of *L. acidophilus* bacteria in yoghurt samples during the maintenance period.

L. acidophilus groups	Count of *L. acidophilus* (log CFU/g) during maintenance period (day)				
	1	7	14	21	28
Control	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]
Simple exposed	8.21±0.1[c]	7.75±0.1[c]	7.65±0.1[c]	7.37±0.0[c]	7.30±0.3[c]
Capsulated exposed	8.61±0.2[c]	7.99±0.0[c]	7.75±0.3[c]	7.45±0.1[c]	7.13±0.1[c]
Simple unexposed	7.13±0.0[b]	6.73±0.3[b]	6.47±0.2[b]	6.26±0.1[b]	6.16±0.2[b]
Capsulated unexposed	7.2±0.1[b]	6.785±0.2[b]	6.45±0.1[b]	6.24±0.0[b]	6.05±0.0[b]

*Dissimilar letters in each column shows significant difference about $P<0.05$.

Table 7. Count of *B. bifidum* bacteria in yoghurt samples during the maintenance period.

B. bifidum groups	Count of *B. bifidum* (log CFU/g) during maintenance period (day)				
	1	7	14	21	28
Control	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]
Simple exposed	7.81±0.0[c]	7.98±0.2[c]	7.72±0.3[c]	7.16±0.06[c]	7.08±0.1[c]
Capsulated exposed	8.25±0.1[c]	8.09±0.4[c]	7.69±0.0[c]	7.46±0.1[c]	7.08±0.1[c]
Simple unexposed	7.02±0.0[b]	6.55±0.6[b]	6.26±0.1[b]	6.14±0.2[b]	6.06±0.1[b]
Capsulated unexposed	7.07±0.1[b]	6.475±0.0[b]	6.53±0.6[b]	6.39±0.3[b]	6.16±0.0[b]

*Dissimilar letters in each column shows significant difference about $P<0.05$.

Table 8. pH content of different treatments of yoghurt samples during the maintenance period.

Yoghurt treatments	pH during maintenance period (day)				
	1	7	14	21	28
Control	4.37±0.06[a]	4.24±0.06[a]	4.14±0.04[a]	4.02±0.05[a]	4.00±0.01[a]
Simple exposed	4.31±0.05[a]	4.21±0.04[a]	4.07±0.06[b]	4.04±0.02[b]	3.89±0.04[a]
Capsulated exposed	4.31±0.06[a]	4.27±0.16[a]	4.12±0.05[b]	4.12±0.01[b]	3.85±0.15[a]
Simple unexposed	4.31±0.06[a]	4.25±0.08[a]	4.04±0.09[b]	3.96±0.03[b]	3.90±0.07[a]
Capsulated unexposed	4.35±0.02[a]	4.24±0.17[a]	4.06±0.05[b]	4.00±0.02[b]	4.00±0.11[a]

*Dissimilar letters in each column shows significant difference about $P<0.05$.

Table 9. Percent of syneresis of different treatments of yoghurt samples during the maintenance period.

Yoghurt treatments	Syneresis (%) during maintenance period (day)				
	1	7	14	21	28
Control	13.90±0.01[a]	20.13±0.32[a]	18.6±0.92[a]	19.2±0.21[a]	18.28±0.18[a]
Simple exposed	13.95±0.07[a]	19.38±0.67[a]	18.5±0.21[a]	19.48±0.74[a]	20.00±0.35[a]
Capsulated exposed	14.50±0.28[a]	18.15±0.92[a]	19.27±0.04[a]	18.65±0.42[a]	21.48±0.60[a]
Simple unexposed	14.25±0.35[a]	19.78±0.18[a]	19.00±0.71[a]	19.55±0.42[a]	19.55±0.14[a]
Capsulated unexposed	14.54±0.02[a]	17.83±0.25[a]	18.43±0.18[a]	18.58±0.04[a]	21.93±0.04[a]

*Dissimilar letters in each column shows significant difference about $P<0.05$.

Sensory Properties

Figure 3 represents the sensory properties of different treatments of yoghurt samples during the storage time. During the present study, encapsulation of probiotics had significant effect on only flavor of yoghurt samples ($P<0.05$). Furthermore, exposure of probiotics to the ZEO caused significant changes in scores given to flavor, texture and overall acceptability of yoghurt samples ($P<0.05$).

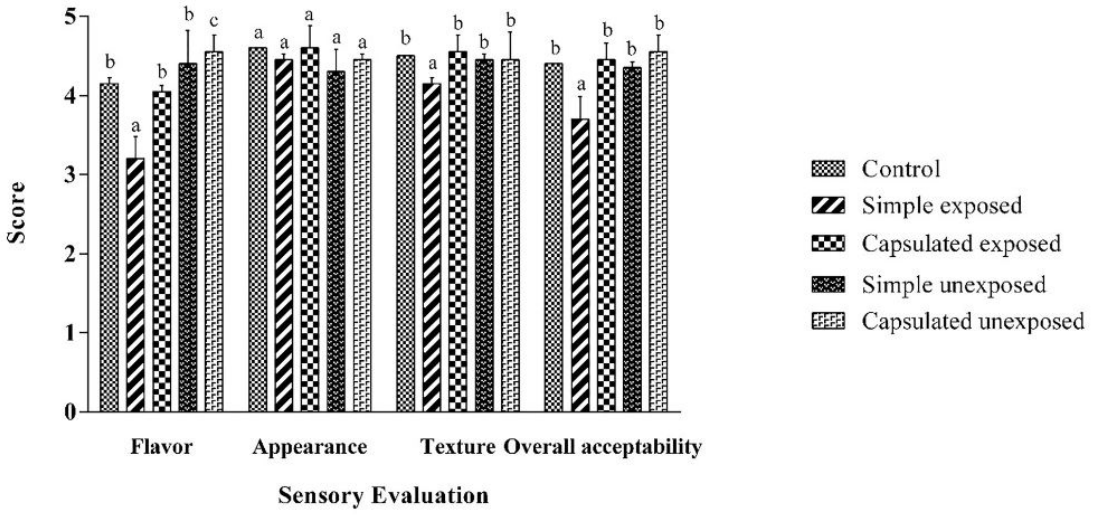

Figure 3. Sensory properties of different treatments of yoghurt samples during the maintenance period. Means in the same line followed by different lower-case alphabets were significantly different. Error bars show standard deviation.

Discussion

It has been suggested that a minimum of 10^6 to 10^7 CFU/g viable cells of probiotics, especially *La-5* and *Bb-12* should be present in a product to provide therapeutic benefits (Lourens and Viljoen, 2001). However, the count of viable cells of probiotic bacteria is decreased during several stages including production, processing and storage and also in the human gastrointestinal tract. Thus, it is essential to increase the viability and survival of *La-5* and *Bb-12* in probiotic dairy products, especially yoghurt to achieve health-related beneficial properties.

The present research was done to study the effect of ZEO and microencapsulation on the viability of *La-5* and *Bb-12* in yoghurt samples and also determine the physicochemical and sensory properties of produced probiotic yoghurt. The results revealed that exposure of bacteria to the ZEO increased the yield of encapsulation and survival of *La-5* and *Bb-12* in both gastrointestinal model and yoghurt matrix. Furthermore, the encapsulation also increased the viability of *La-5* and *Bb-12* in both gastrointestinal model and yoghurt matrix. However, exposure of bacteria to ZEO and also encapsulation did not cause significant changes in the pH content of the yoghurt samples. Moreover, exposure of bacteria to ZEO caused significant decrease in the syneresis percent of yoghurt samples. The encapsulation of bacteria and also their exposure to ZEO caused an increase in the scores given to the sensory properties. However, yoghurt samples of control group delivered the highest sensory properties. Put together, exposure of *La-5* and *Bb-12* to the ZEO and also their encapsulation caused positive changes in the physicochemical and sensory properties of the yoghurt samples and also increased their viability in both yoghurt matrix and gastrointestinal model. Similar investigations have been conducted in this field. Ghaleh Mosiyani *et al.* (2017) reported that exposure to basil and savory extracts caused significant increase in the viability of *Lactobacillus paracasei* ssp. *paracasei* during the storage time in probiotic yoghurt. The mean scores given to taste, odor, texture, color and overall acceptance of yoghurt samples treated with basil and savory extracts were higher than other treatments. This finding was also similar to those reported by Michael *et al.* (2015) and Sarabi-Jamab and Niazmand (2009). Rezazadeh *et al.* (2015) reported that vanillin caused significant increase in the viability of *La-5* and *Bb-12* in the yoghurt samples compared to the control group. They also showed that yoghurt samples treated with probiotics and vanillin had higher scores given to taste, thickness and flavor sensory properties compared to the control group. Marhamatizadeh (2015) reported that exposure of *La-5* and *Bb-12* to garlic and dill extracts caused significant increase in their survival during the storage time of yoghurt samples. Additionally, he showed that taste, color, and insolubility properties of yoghurt samples treated with garlic and dill extracts were significantly better than the control group.

We found that the total population of *La-5* and *Bb-12* decreased significantly in the last days of storage time, which can be due to the accumulation of lactic acid produced by the starter culture, leading to a reduction in pH and an increase in acidity (Joung *et al.*, 2016). Increase in Eh and the hydrogen peroxide concentration coming from the metabolic activity of *La-5* and *Bb-12* can lead to a reduction in bacterial counts during the storage time. Reversely, the presence of certain chemical components such as thymol, alpha-terpineol, carvacrol, linalool and gamma-terpinene increased the growth and survival of *La-5* and *Bb-12*. It has been documented that phenolic components of natural EOs play a stimulating role and enhance the growth of the starter culture of yoghurt and probiotics (Oh *et al.*, 2016; Marhamatizadeh, *et al.*, 2013). The effect of antioxidant compounds on fermentation time and survival of probiotics during yoghurt production has been studied (Amirdivani and Baba, 2011; Felix *et al.*, 2017). The ZEO could act as supplementary energy source or exert antioxidant effects. Moreover, plants EOs contain adequate amounts of vitamins and carbohydrates that guarantee the growth and survival of *La-5* and *Bb-12* in yoghurt. Conversely, lack of growth-stimulating agents, such as ZEO is the reason for the remarkable reduction of probiotic counts in control yoghurt samples. Thus, ZEO can function as prebiotic, a complex of polysaccharide pectin and pectic-oligosaccharide. It

can also promote the growth rate of certain probiotics. Similar findings were also reported for the exposure of probiotics to mint, thyme and garlic (Simsek *et al.*, 2007), Ziziphora (Khodaparast *et al.*, 2007), Chamomile (Marhamatizadeh *et al.*, 2012) and barberry (Hassani *et al.*, 2016). Jimborean *et al.* (2016) found that the yoghurt incorporated with orange EOs increases the viability of the lactic acid bacteria depending on the biologically active compounds coming from the orange peels.

In addition to yoghurt matrix, encapsulation and exposure of bacteria to the ZEO caused significant effect on the survival of probiotics in the gastrointestinal model. The success of probiotic survival in gastric conditions is predominantly due to alginate gel, which provides the appropriate protection to the probiotic cells. Additionally, chitosan, a positively charged polyamine, constitutes a semipermeable membrane around alginate, a negatively charged polymer. This membrane is not dissolved in the presence of calcium ions chelators or anti-gelling factors and thus increases the stability of the gel and constructs a barrier to the cell release (Smidsrod, 1990). The positive effects of probiotic bacteria encapsulation using alginate and chitosan on their survival and viability was also reported previously (Mandal and Singh, 2006; Abbaszadeh *et al.*, 2014).

Exposure of probiotics to the ZEO caused significant decrease in the pH content of yoghurt samples. The reason for this is the fact that yoghurt fermentation with the herbal extracts increased the metabolic activity of the yoghurt bacteria, thus elevating the yoghurt acidity due to the production of organic acids by lactic acid bacteria and then caused significant decrease in the pH content (36). The pH also decreased during the storage time because as the storage time increased, the lactose fermentation by the starter and probiotic proceeded, and pH decreased due to the accumulation of organic acids such as lactic acid and formic acid (29, 33). Omidvar *et al.* (2014) reported similar findings about the pH content of yoghurt samples treated with ZEO. They revealed that ZEO caused significant decrease in the pH content of samples. Similar findings were also reported by Samedi and Charles (2019), Ghasemnezhad *et al.* (2016), Shahdadi *et al.* (2014) and Yangilar and Yildiz (2017). However, different findings have been reported in the other researches. Chaikham (2015) reported pH in probiotic yoghurt samples to change from 4.45–4.48 to 4.30–4.36 on day 0 and 30, respectively while in Ghalem and Zouaoui study (2013a), pH ranged from 4.08 to 4.66 for yoghurt sample fortified with Chamaemelum spp. extract and from 4.52 to 4.61 for the sample enriched with Lavandula spp. EOs. Ghalem and Zouaoui (2013b) reported pH to be stable in the yoghurt samples fortified with *Rosmarinus officinalis* EO during the storage time while that of the control sample decreased significantly. Differences may be due to the variance in the applied EOs, applied probiotic bacteria and also studied probiotic samples.

Syneresis is controlled by the balance between attraction and repulsion forces within the casein network and the rearrangement capacity of the network bonds (Giroux *et al.*, 2014). Syneresis or whey separation may sign low quality when its rate is high and be counted among the quality parameters for yoghurt and the most important factors affecting consumer's acceptance. In this study, syneresis decreased significantly by the exposure of probiotic bacteria to the ZEO (P<0.05). This effect may be explained by the structural difference in the gels induced by phenolic compounds. Polyphenols may increase rearrangements, which would results in larger pore size in the gel matrix which is associated with higher syneresis. Interactions between phenolic compounds and yoghurt proteins allow water not connect strongly to the network proteins (Han *et al.*, 2011). The storage time was shown to affect the syneresis rate in the yoghurt samples based on

the contracting effect resulting from low pH on casein particles and thus increasing the resistance of yoghurt to syneresis. However, syneresis had irregular procedure in some studied days of storage time.

The encapsulation and exposure of probiotic to the ZEO caused some improvement in the sensory properties, especially flavor, appearance and texture of yoghurt samples. Yangilar and Yildiz (2017) reported that yoghurt samples treated with ginger and chamomile EOs had significantly higher sensory scores ($P<0.05$) for the color and appearance, flavor, texture, syneresis, odor, acidity and general acceptability which was similar to our findings. Joung *et al.* (2016) stated that yoghurt may carry plant extracts well, which can improve the organoleptic properties of yoghurt like complemented sourness, increased bitterness, favored flavor, viscosity, and texture. It is important to determine the characteristics of yoghurt texture in order to ensure the development of products and processes, quality control and consumers' acceptability. Moritz *et al.* (2012) found that application of cinnamon EO caused significant increase in the scores given to flavor, color and overall acceptability. The ZEO is widely used as a flavouring agent in yoghurt amongst the Iranian people. Thus, it is not surprising that the scores given to flavour of treated yoghurts was higher than the other groups. Shahdadi *et al.* (2015) reported that probiotic yoghurt samples treated with mint (*Mentha spicata*), bee balm (*Mentha longifolia*), eucalyptus (*Eucalyptus camaldulensis*) and ziziphora (*Ziziphora tenuior* L) EOs had the highest scores for odour, taste, color, texture and overall acceptability. Production of lactic acid and aromatic compounds such as acetaldehyde, acetone, acetoyin and diacetyl could define our results.

Conclusion

To put it in a nutshell, the present study identified the effects of encapsulation and exposure to sublethal dose of ZEO on the survival and viability of *Bb-12* and *La-5* and physicochemical and organoleptic characteristics of produced yoghurts both in food matrix and gastrointestinal model. The sub-lethal dose of ZEO can be used as an ingredient in probiotic yoghurt containing *La-5* and *Bb-12* to ensure the survival and viability of probiotics and improve some of the physicochemical and sensory properties of yoghurt. Additionally, encapsulation of bacteria with alginate and chitosan was determined as a practical method to improve the survival and viability of bacteria in both yoghurt samples during the storage time and also gastrointestinal model. The yoghurt samples treated with encapsulated and exposed probiotics had better pH, lower syneresis and higher scores given to sensory properties. Additionally, encapsulated and exposed probiotics had higher survival and viability during the storage time of yoghurt samples and also in gastrointestinal model. In keeping with this, from a sensory stand point no differences were found between the samples. The production of supplemented probiotic yoghurt with ZEO and encapsulated probiotics is feasible in industrial and consumer point of views. However, further researches are required to find more information about the probiotic yoghurt containing ZEO and encapsulated *La-5* and *Bb-12*.

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Conflict of Interest

The authors declared no conflict of interest.

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مجله طب دامی ایران، ۱۳۹۹، دوره ۱۵، شماره ۲، ۲۳۴-۲۵۳

مطالعه اثر استرس اسانس *کاکوتی کوهی* و ریزپوشانی با آلژینات –کیتوزان بر زنده‌مانی *لاکتوباسیلوس اسیدوفیلوس* و *بیفیدوباکتریوم بیفیدوم* و خصوصیات حسی و فیزیکوشیمیایی ماست پروبیوتیک

نفیسه علی قاضی، نگین نوری*، حسن گندمی، افشین آخوندزاده بستی

گروه بهداشت مواد غذایی، دانشکدهٔ دامپزشکی، دانشگاه تهران، تهران، ایران

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زمینه مطالعه: پروبیوتیک‌ها پس از عبور از معده باید در تعداد کافی زنده بمانند و یکی از اصلی‌ترین استرس‌هایی که سویه‌های پروبیوتیکی باید تحمل کنند، وجود مواد نگهدارنده در مواد غذایی مانند اسانس‌ها است. به‌منظور برقراری تعادل بین قابل بودن خواص حسی و اثر ضد میکربی اسانس‌ها، استفاده از غلظت تحت کشنده آن‌ها توأم با سایر نگهدارنده‌ها پیشنهاد می‌شود.

هدف: هدف از این مطالعه ارزیابی اثر استرس مواجهه با غلظت تحت کشنده اسانس *کاکوتی کوهی* بر زنده‌مانی *لاکتوباسیلوس اسیدوفیلوس* و *بیفیدوباکتریوم بیفیدوم* میکروکپسوله و خصوصیات فیزیکوشیمیایی و حسی ماست پروبیوتیکی در طی ۲۸ روز نگهداری می‌باشد. علاوه بر این، بقای پروبیوتیک‌ها نیز در شرایط دستگاه گوارش مورد بررسی قرار گرفت.

روش کار: غلظت تحت کشنده و کشنده اسانس *کاکوتی کوهی* برای *لاکتوباسیلوس اسیدوفیلوس* و *بیفیدوباکتریوم بیفیدوم* تعیین شد. 10^9 cfu/mL از هر دو پروبیوتیک در معرض غلظت تحت کشنده اسانس *کاکوتی کوهی* در محیط MRS براث به مدت ۲ ساعت قرار گرفتند و سپس با آلژینات و کیتوزان میکروکپسوله شدند. ابتدا، زنده‌مانی پروبیوتیک‌های کپسوله شده در شرایط معدی روده‌ای تخمین زده شد. پس از تهیه ماست و تلقیح پروبیوتیک‌های مواجهه شده با غلظت تحت کشنده اسانس به دو صورت میکروکپسوله و غیر میکروکپسوله، شمارش آن‌ها انجام شد. در نهایت، ویژگی‌های فیزیکوشیمیایی و حسی پروبیوتیک‌ها در ماست اندازه‌گیری شد.

نتایج: تیمول ۷۰/۴۱ درصد، آلفا ترپینول ۷/۳۱ درصد و کارواکرول ۳۹/۵ درصد بیشترین اجزای مورد استفاده در اسانس بودند. غلظت کشنده اسانس کاکوتی کوهی برای *لاکتوباسیلوس اسیدوفیلوس* و *بیفیدوباکتریوم بیفیدوم* به ترتیب ۱۷۵۰ و ۱۵۰۰ پی‌پی‌ام بود. میکروکپسوله کردن و مواجهه پروبیوتیک‌ها با غلظت تحت کشنده اسانس به‌طور معنی‌داری بقای پروبیوتیک‌ها را در شرایط معدی- روده‌ای و ماست طی ۲۸ روز نگهداری افزایش داد. همچنین کپسوله کردن و مواجهه پروبیوتیک‌ها با غلظت تحت کشنده باعث تغییر معنی‌داری در pH نمونه‌های ماست شد ($P>0/05$). از طرف دیگر، آب‌اندازی در همه نمونه‌ها افزایش یافت ($P>0/05$). گروه مواجهه یافته با غلظت تحت کشنده اسانس امتیاز کمتری در طعم را به خود اختصاص دادند. با این وجود، بین گروه‌های مواجهه یافته و سایر گروه‌ها از نظر طعم، بافت و پذیرش کلی تفاوت معنی‌داری وجود داشت ($P>0/05$).

نتیجه‌گیری نهایی: مواجهه با غلظت تحت کشنده اسانس *کاکوتی کوهی* می‌تواند به عنوان پری‌بیوتیک در ماست حاوی پروبیوتیک‌ها سبب بهبود بقا و زنده ماندن پروبیوتیک‌های میکروکپسوله شده و هم‌چنین سبب ارتقا برخی از خصوصیات فیزیکو شیمیایی و حسی گردد.

واژه‌های کلیدی: اسانس کاکوتی کوهی، *بیفیدوباکتریوم بیفیدوم*، *لاکتوباسیلوس اسیدوفیلوس*، ماست پروبیوتیک، میکروکپسولاسیون

نویسندهٔ مسئول: نگین نوری ، گروه بهداشت مواد غذایی، دانشکدهٔ دامپزشکی، دانشگاه تهران، تهران، ایران. ایمیل: nnoori@ut.ac.ir