Effect of *Bunium persicum* essential oil, NaCl, Bile Salts, and their combinations on the viability of *Lactobacillus acidophilus* in probiotic yogurt

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RESEARCH ARTICLE

Abstract

The probiotic yogurt, with additional essential oil researches, has increased recently. *Bunium persicum* Boiss is a critical medicinal wild growing plant in Iran dry areas. In this study, *Lactobacillus acidophilus* was exposed to stress with *B. persicum* essential oil (BEO), NaCl, bile salts, and their combinations by 50 minimum inhibitory concentration (MIC) and then inoculated to the yogurt samples, which were stored at 4°C for 28 days. Independent parameters in this article are BEO 500 and 1000 ppm that the physicochemical, sensory properties, and *L. acidophilus* of viability in yogurt samples were assessed within the time. A steady increase in syneresis percentage and acidity was observed, while pH values were reduced in all samples. The *L. acidophilus* survival decreased during storage time in all treatments. In addition, sensory scores showed a reduction trend in the samples. The survival rate of probiotic bacteria is also impacted by redox potential. Increasing the oxidation and resuscitation potential and increasing the hydrogen peroxide concentration due to the bacteria metabolic activity are among the factors that reduce the probiotic bacteria population in yogurt during storage. The most viability of *L. acidophilus* under stress with BEO compared with other stress treatments may be due to slight changes in pH during this period than in the stress treatments. Generally, it can be argued that the usage of BEO, NaCl, and bile salts at a MIC of 50% in yogurt stored in the refrigerator for 21 days provided a suitable environment for the storage and transmission of *L. acidophilus*, as recommended to the consumer and, therefore, the current results confirmed that the addition of probiotic and these materials improved the physicochemical and sensory characteristics of yogurt.

Keywords: *Bunium persicum*; *L. acidophilus*; stress; Yogurt

Introduction

Food producers faced opportunities and challenges due to growth in the functional foods market, and the same situation happened for academic departments to achieve the customer confirmation during the recent decade (Mousavi *et al.*, 2019). One of the worldwide favorite probiotic products in the dairy industry is yogurt. Food does not only provide nutrients but is also an active ingredient in bioactive substances. Certain types of foods, which provide energy and improve consumers' health, are called functional foods (Kocer and Unal, 2018). In addition to its beneficial health properties, the incorporation of the prebiotic lactobionic acid (LBA) in fermented

dairy products can provide a technological advantage due to its gelling capacities (Cristina *et al*., 2018). Probiotic strains are microorganisms that bring about many beneficial effects for their host health. Moreover, three prerequisites for probiotic selection include the viability of the microorganism, sufficient administration, and at least one beneficial health effect (Rezaei *et al*., 2020). Probiotic products are important in processed foods (Yangilar and Yildiz, 2018). It is proven that probiotic strains of lactic acid bacteria (LAB) can control gastrointestinal tract (GIT) microbiota by inhibiting the opportunistic bacteria growth, therefore, to control enteric pathogens, increment in probiotic strain activity in GIT is considerable (Mousavi *et al*., 2019). The activity of probiotics is increased in the presence of selected prebiotics. Therefore, it is suggested that the combination of probiotics and prebiotics (synbiotics) can give improved health benefits. The prebiotics enhances the probiotics' beneficial activity, but at the same time, nondigestible and fermentable prebiotics such as fructans, inulin, and oligofructose play an important role in lowering the cholesterol levels in the blood. These inhibit the build-up of free cholesterol as plagues in arteries (Farkhandah *et al*., 2019). Various suitable strains of Lactobacilli and Bifidobacteria exist for human use (Marhamatizadeh and Ramezanian, 2016). The produced organic acids, especially lactate, by LAB during fermentation lead to a reduction in the pH, and Lactobacilli metabolic activity will lead to pH decrement till critical limit (Hashemi *et al*., 2018). According to several studies in this regard, to have the best therapeutic effects, it is needed to present an optimum number of probiotics, which is the "therapeutic minimum" and the amount is 105–106 CFU/g or mL of the product at consumption time and 108 CFU/g to have it in the gut (Tharmaraj and Shah, 2004) and the live cells (Sahadeva *et al*., 2011). Many factors such as bacterial type, pH, product acidity, heat-dependent processes (incubation and storage temperature), and growth factors influence the probiotic bacteria survival. Besides these, the probiotic viability of fermented products is also low due to low pH and high acidity, so the critical factor for these bacteria survival is to have high acidity along with low pH parameters (Kitazawa *et al*., 2001). However, during the food production process and digestion, the number of living cells is reduced in the small intestine due to stresses such as heat, cold, osmotic pressure, stomach acid, and bile salts (Sahadeva *et al*., 2011). Beneficial effects such as mucosal barrier increment, vitamin B synthesizing, cholesterol serum decrement, increasing the immune system, intestinal inflammation control, pathogenic microorganisms affecting control, diarrhea reduction, and antimutagenic activity increment can be obtained by consuming probiotics (Hashemi *et al*., 2017). Prebiotic could be appropriate for probiotic strain viability during the production period and product storage and protecting them while passing the GIT to reach the colon (Kocer and Unal, 2018). Genes can be activated to withstand stress (Maragkoudakis *et al*., 2006). Many ingredients have been added to yogurt in recent years, including *Primula vulgaris* (Lee *et al*., 2007), *Mentha piperita*, and *Ziziphora clinopodioides* (Sarabi-Jamab and Niazmand, 2009) to improve the nutritional, medicinal, and sensory properties and maintenance of yogurt. There are conflicting reports about the effecting of essential oils on the sensory properties so probiotic survival in yogurt, where some studies have shown a decrease in the sensory qualities of the yogurt (Moritz *et al*., 2015; Shahdadi *et al*., 2015), improved product properties sensory (Azizkhani and Parsaeimehr, 2018; Shahdadi *et al*., 2015), no adverse effect on probiotic activity (Azizkhani and Parsaeimehr, 2018; Sarabi-Jamab and Niazmand, 2009), and a negative impact on the activity and survival of probiotics (Azizkhani and Parsaeimehr, 2018). On the other side, the minimum inhibitory concentration (MIC) of plant essential oils against probiotics is high, while at the value of concentrations, the essential oils have inhibitory effects on pathogens. Therefore, the use of essential oils in low doses can kill pathogens without harming the probiotics (Calsamiglia *et al*., 2007). However, the probiotics' survival in the production, maintenance, and consumption of yogurt is unclear because these bacteria are subject to various stresses. Therefore, the viability of commercial probiotic strains such as *Lactobacillus acidophilus* when exposed to NaCl and bile salts under different conditions should be evaluated. Many studies were performed to evaluate the probiotic survival strains against bile salts in vitro (Del Piano *et al*., 2006). However, there were some differences between the observations made in vitro and in vivo (Sahadeva *et al*., 2011). A combination of herbal products and probiotics can lead to tremendous major antimicrobial therapy. Probiotic microorganisms' viability may be impacted due to the antimicrobial feature of herbs. Studies showed that herbs could enhance probiotics and pathogens inhibition (Be *et al*., 2009; Sutherland *et al*., 2009). Using herbal essential oil extract would significantly affect product properties, such as protein nutrition and structures (Keshavarzi *et al*., 2020). According to the study of Massoud *et al*. (2020) *Rosmarinus officinalis* essential oil (REO) has a considerable potential to deliver *Bifidobacterium bifidum* in a sufficient population in yogurt, as well as starter cultures. The lowest pH and highest acidity were found in the samples supplemented with 1% REO. Besides, the optimal viscosity and sensory scores of probiotic yogurts were found in the samples supplemented with 1% REO. The statistical analyses also revealed that during the refrigerated storage, the pH, sensory properties, bacterial counts, and viscosity reduced, while the acidity values increased (Massoud *et al*., 2020). In addition, in two separate studies conducted by Marteau *et al*. in 1997 and Lin *et al*. in 2006, the results presented no remarkable differences between the in vitro and in vivo observations. Therefore, the article's objective

was about the effects of *Bunium persicum* Boiss essential oil (BEO), NaCl (NC), and bile salts (BS) on *L. acidophilus* survival and physicochemical and sensory properties of produced yogurt.

Materials and Methods

Materials

BHI agar and BHIB broth medium were obtained from Sigma USA, MRS broth and MRS agar medium from Merck, and NaCl and bile salts from Sigma USA. Cow milk was obtained from Pegah (Tehran Plant), and Yogurt Commercial Ingredients: Duplex Express (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) were received from Chr. Hansen, Ltd., Denmark. All the processes to examination are showed in Figure 1.

Preparation of plant, essential oil, and its analysis

The *B. persicum* was collected from Kerman province of Iran in the summer, and its scientific name was confirmed by the Iranian National Plant Protection Research Institute in Tehran. The essential plant oil was extracted by water distillation and then analyzed by gas chromatography attached to a mass spectrometer (Model HP-6890, USA). For this purpose, HP-5MS capillary column with 30 m length and 0.25 mm inner diameter and 0.32 µm inner layer thickness was used. The temperature program was used at 60 to 265°C, with a gradual increase of 2.5°C, and finally, the column was maintained at 265°C for 30 min. The injection room temperature was 250°C, and the helium carrier gas passed through the tube at a speed of 1 mm/min (Mehdizadeh *et al*., 2019). Finally, the essential oil components were identified by ionization energy extraction time using FID inductor with an electrical

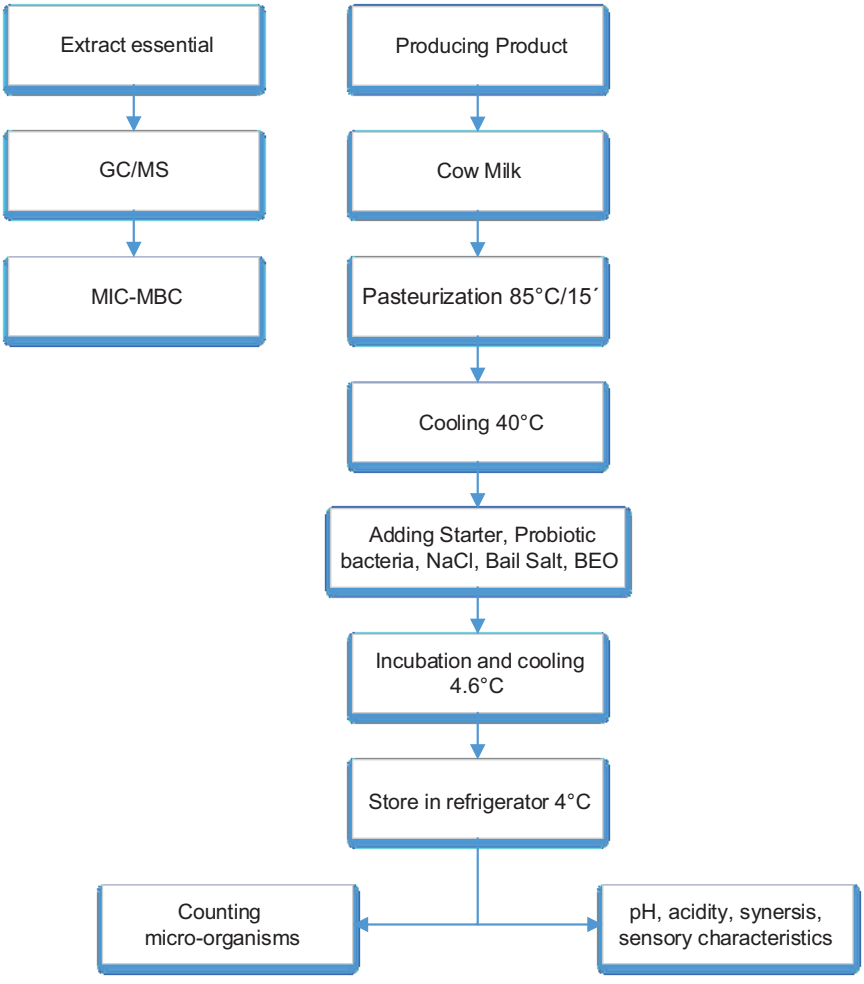

Figure 1. Process to examination flowchart.

capacity of 70 eV and ionization source temperature of 250°C (Marriott *et al*., 2001).

Preparation of inoculum

L. acidophilus is a popular strain in probiotics. Its usage of probiotic products is growing around the world (Tornuk *et al*., 2014). The strain of bacteria used in this article was *L. acidophilus* (ATCC4356), obtained from the institute of microbial collections of Iran. It was kept in a laboratory as a glycerol stock at –70°C and transferred in Brain Heart Infusion (BHI) broth at 37°C without shaking. Working cultures were prepared from stock cultures by two sequential transfers (1% inoculum) in BHI broth at 37°C for 18 h. *L. acidophilus* inoculated from working cultures to tubes of BHIB. Then, the secondary subculture was incubated in the same duration and temperature, and broth culture was adjusted to an optical density (OD) (absorbance) of 0.1 at 600 nm, using a Spectronic 20 spectrophotometer (Varian, USA). This adjustment gave a cell concentration of 2.2×1010 CFU/mL for *acidophilus* as detected in preliminary trials. The estimation of cells number in suspensions was done by counting the colonies at 37˚C after 24 h of incubation and duplicate plating serial dilutions from 10-fold on BHI agar.

Determining the minimum inhibitory concentration of Bunium persicum Bioss essential oil, NaCl, and bile salt

To determine the MIC, 96-well plates with a volume of 300 µL were used. Then 100 µL of essential oil, NaCl, and bile salts were transferred to each well, and 20 µL of bacterial suspension was added (final concentration of bacteria was 5×10^6 CFU/mL). The contents of each well were mixed with a Shaker Plate Reader for 2 min. The optical absorption was then read using a plate reader at 0 h at 630 nm. Plates incubation took 24 h at 35°C, and after visualization of turbidity or nonturbidity in the well, they were visualized and light absorbed by a plate reader at the mentioned wavelength.

Adaptation and challenge conditions

Cultures were grown to the mid-exponential growth phase. Bacterial cells were collected via centrifugation (Hettich, German) and suspended again in fresh BHIB (nonadapted control culture). The inoculation dose of *L. acidophilus* was 1×1010 CFU/mL. Adaptation (120 min) was performed in the same medium at 37°C (i) with 700 mL in 500 cc medium including 50% DMSO (BEO), (ii) 10 g per 100 mL in Mueller Hinton broth medium (NC), (iii) 0.01 g per 100 mL in Mueller Hinton broth medium (BS), (iv) 700 mL in 500 cc medium included 5% DMSO plus 10 g per 100 mL in Mueller Hinton broth medium (BEONC), (v) 700 mL in 500 cc medium included 50% DMSO plus 0.01 g per 100 mL in Mueller Hinton broth medium (BEOBS), (vi) 10 g per 100 mL plus 0.01 g per 100 mL in Mueller Hinton broth medium (NCBS), and (vii) 700 mL in 500 cc medium included 50% DMSO, 10 g per 100 mL, and 0.01 g per 100 mL in Mueller Hinton broth medium. After centrifugation, adapted and nonadapted cells were inoculated to the sterilized yogurt (108 per mL). Measuring colony-forming unit capacity (CFU) for samples (0.01mL) was done by removing at the desired intervals of days (0, 7, 14, 21, and 28). Plates were incubated before counting colonies at 37°C for 48 h. By challenge treatment to CFU at time zero, survival was specified as the ratio of CFU. In all experiments that have been done at least three times, each evaluation is the duplicate plating average.

Measurement of physicochemical properties of probiotic yogurt

Measurement of pH and acidity

The increment of yogurt bacteria's metabolic activity and acidity results from fermenting yogurt with essential oils, which leads to acidity increment of treatments compared with the control sample due to producing organic acids of lactic acid bacteria. The reduction in pH values is by the growth of the lactic bacteria. Maltose, glucose, and fructose, respectively, had the highest consumption rate (Shokoohi *et al*., 2015). The acidity increased, and pH decreases gradually in all treatments because of the accumulation of acids such as lactic acid and formic acid (Keshavarzi *et al*., 2020). The acidity of samples as specified by the titration method is based on the percentage of lactic acid. 10 mL of the sample was titrated in the existence of phenolphthalein against N/10 NaOH. pH values were measured with a digital pH meter (Shahdadi *et al*., 2015).

Syneresis

First, to measure syneresis's amount, 25 g of yogurt sample was weighed in centrifuge tubes and centrifuged at 350 x. g and 10°C for 30 min. The liquid that appeared on the tube was removed, and again the tubes were weighed. The amount of watering was reported as 100 grams of yogurt lost weight (Shahdadi *et al*., 2015).

Sensory analysis

Texture, flavor, and overall acceptability of probiotic-yogurt samples were analyzed after 28-day at 4°C storage. The characteristics of texture, flavor, and overall acceptability of the samples were evaluated by 10 trained panelists using the five-point hedonic method. The very good (very satisfied) score was considered 5, good (satisfactory) score of 4, average (acceptable) score of 3, poor

(unacceptable) score of 2, and a very poor (unacceptable) score of 1. To show the importance of flavor in our sensory attributes, the sample is considered unacceptable in terms of the overall evaluation (Shahdadi *et al*., 2015).

Statistical analysis

Data were inspected utilized Completely Randomized Design (CRD). The Tukey test in SAS version 9 software performed the comparisons of data mean. Three replications were performed for each sample. Two-way ANOVA was used to determine the significance of the nonsignificance of data ($P < 0.01$).

Results and Discussion

Chemical composition of Bunium persicum Boiss essential oil

GC–MS analysis of the essential oil led to identifying 12 different compounds, representing 100% of the total oil (Table 1).

The main components were Carvone (26.03%), Propanal, 2-methyl-3-phenyl (18.59%), Gamma-Terpinene (17.43%), and Limonene (12.84%). It would also be noteworthy to point out that the composition of any plant essential oil studied is influenced by the presence of several factors, such as local, climatic, seasonal, and experimental conditions (Shahsavari *et al*., 2008). Abduganiew *et al*. (1997) analyzed the BPEO originating from Tajikistan, and they detected 22 compounds, including p-mentha-1, 4-dien-7 al, γ-terpinene, β-pinene, and cumin aldehyde (Abduganiew *et al*., 1997) and Mahmoudvand *et al*. analyzed BPEO that grow in the Jiroft, Kerman, Iran. The main components were g-terpinene (46.1%), cumin aldehyde (15.5%), r-cymene (6.7%), and limonene (5.9%) (Mahmoudvand *et al*., 2016).

Table 1. Results of *Bunium persicum* Boiss essential oil.

No.	Name	Area%
1	α-Pinene	0.89
2	Sabinene	0.69
3	β-Pinene	1.79
4	β-Myrcene	0.62
5	Cymene	9.62
6	Limonene	12.84
7	Gamma-Terpinene	17.43
8	3-Cyclopentylcyclopentan-1-one	0.98
9	Propanal, 2-methyl-3-phenyl-	18.59
10	Carvone	26.03
11	1-Isopropylidene-3-n-butyl-2-cyclobutene	3.63
12	(R)-(+)-1-Phenyl-1-propanol	6.89

MIC results

The MIC usually shows the antimicrobial effectiveness of a compound. The lowest compound concentration could inhibit organism growth. The MIC was described as "the lowest concentration, which resulted in maintenance or reduction of inoculum viability" over a 24-h contact time (Mann and Markham, 1998). The MIC of BEO, NC, and BS were 1, 4, and 0.3%, respectively.

Viability of *L. acidophilus*

The viability decrement ($P < 0.01$) of *L. acidophilus* was observed during storage in all samples (Figure 2).

According to Figure 2, bypassing storage time, the L. *acidophilus* population in all the probiotic yogurt samples decreased, but the fall of survival rate in control and BEO treatments was less than that of the other treatments ($P < 0.01$). Based on a comparison of the population of *L. acidophilus* among all treatments at each point in time, it was found that the control and BEO treatments had a higher number of probiotic *L. acidophilus* throughout the days of storage compared with the stress treatments ($P < 0.01$). In addition, no considerable difference was observed between control and BEO treatments in *L. acidophilus* survival ($P > 0.01$). Among the stress treatments, the survival of the *L. acidophilus* was considerably higher and lower in the treatments with BEO and the stress of the BEONCBS, respectively ($P < 0.01$). There was no considerable difference between the survival of the *L. acidophilus* in the treatments under NC and BC alone and combination with BEO throughout the days of storage ($P > 0.01$). In addition, the stress treatment with NCBS after treatment with BEO had the lowest number of *L. acidophilus*. After 28 days of storage, the control treatment (5.69 ± 0.02 log CFU/mL) and the stress treatment with BEONCBS (4.47 ± 0.04 log CFU/mL) were assigned the highest and the lowest number of *L. acidophilus*, respectively. In the production of probiotics, the probiotic bacteria survival in yogurt and other similar products during storage until consumption is a critical factor (Azizkhani and Parsaeimehr, 2018). In this study, the *L. acidophilus* population in probiotic yogurt was considerably reduced after 4 weeks of storage at 4°C. In the control treatment, the *L. acidophilus* population was reduced from 7.58 ± 0.01 log CFU/mL on the first day of the storage period to 5.69 ± 0.02 log CFU/mL on the 28th day of the storage period. The *L. acidophilus* population was less than 5 logs CFU/mL after 28 days of storage for all the stress treatments (except for BEO, which was 5.66 ± 0.01 log CFU/mL). In the control treatment, the *L. acidophilus* bacteria amount slightly increased during the first 7 days of storage. Other researchers have reported an increment in the population of *L. acidophilus* bacteria in the

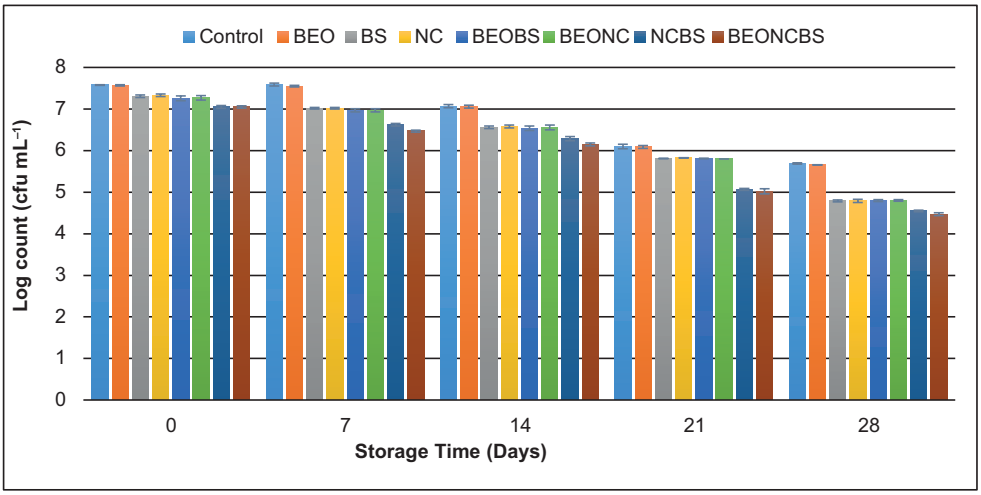

Figure 2. Effects of *Bunium persicum* essential oil (BEO), NaCl (NC), bile salts (BS), and their combinations on the survival of *L. acidophilus* ATCC-4356 (log CFU/mL). Deviation bars designate the standard error of the method (n = 3).

probiotic yogurt during the first 7 days of storage (Cruz *et al*., 2012; Sarabi-Jamab and Niazmand, 2009). There were no considerable differences between the control and BEO and NC and BC treatments alone and in combination with BEO during the period of storage. This may be due to their bacterial resistance to pH values above 4. *L. acidophilus* had resistance to acidic conditions, and it could survive up to pH = 4.05 ± 0.05. *L. acidophilus* can grow at pH = 4 and also titratable acidity should be more than 0.6% (Shah, 2000). The population decrement in the *L. acidophilus* bacteria during storage may be related to the organic acids cumulation due to growth and fermentation (Cruz *et al*., 2012). Probiotics are microorganisms that are alive and can produce substances that encourage the reproduction of other microorganisms. However, these microorganisms' effects depend on the strain they belong to and the dose in the product in which they are found. On the other hand, the survival, growth, and viability of these microorganisms are affected by probiotic food production stages (Gunes and Gultekin, 2018). The *L. acidophilus* bacteria viability in the control treatment was higher than other treatments during the period of storage. Among the stress treatments, the number of *L. acidophilus* was considerably higher in the treatment with BEO than the other treatments ($P < 0.01$). The most viability of *L. acidophilus* under stress with BEO compared with other stress treatments may be due to slight changes in pH during this period than in the stress treatments (Azizkhani and Parsaeimehr, 2018). Other studies mentioned reducing *L. acidophilus* viability in probiotic yogurt containing essential oils during storage (Azizkhani and Parsaeimehr, 2018; Lee *et al*., 2007; Shahdadi *et al*., 2015). Studies showed that *M. piperita* essential oil and *peppermint* decreased the activity of *L. acidophilus* in probiotic yogurt (Sarabi-Jamab and Niazmand, 2009). One of the important criteria in selecting lactic acid bacteria for use as probiotics is their resistance to NaCl and bile salt concentration, which are the essential requirements for small intestine bacteria metabolic activity increment (Sutherland *et al*., 2009). The small and large intestines of humans and animals contain relatively large bile acids that can inhibit the growth or kill many bacteria. Therefore, probiotic bacteria must grow at bile concentrations of 0.15–0.30 to be effective (Šušković *et al*., 2000). In the present study, the *L. acidophilus* viability in stress treatment containing 0.15% BS was 5.81 ± 0 CFU/mL up to 21 days of storage as defined in the range of 10^5–10^6 CFU/mL for food. The resistance of some strains to bile salts is attributed to their ability to hydrolyze bile salts, which reduces their toxicity and adverse effects (Amraii *et al*., 2014). After 21 days of storage, the *L. acidophilus* bacteria population was decreased, and the survival rate of *L. acidophilus* was less than the permissible limit. The main reason for the fall of *L. acidophilus* is cellular homeostatic abnormalities where they were exposed to bile salts. The cell death mechanism of probiotic strain was related to cell membrane lipids and protein breakdown (Amraii *et al*., 2014; Sahadeva *et al*., 2011). Indeed, bile salts kill microorganisms by disrupting the structure of the cell walls. Therefore, resistance to bile salts is an essential feature of probiotics that preserve their viability and activity in the small intestines. Probiotics neutralize their effects by producing bile salt-hydrolyzing enzymes (Sahadeva *et al*., 2011). Different salt concentrations impact the probiotic strains' survival rate, especially at the industrial level (Gandhi and Shah, 2015), and also, the *L. acidophilus* viability was decreased when exposed to high concentrations of salt during the storage period (Gandhi and Shah, 2015). The results obtained by Massoud and Sharifan showed a considerable decline in the *L. acidophilus* activity after exposure to the essential oils (EOs) of

Z. clinopodioides and *M. piperita* in yogurt. The chemical composition of EOs, their phenolic profiles, and secreted metabolites by the starter cultures could cause various effects on bacterial growth. Notably, phenolic compounds could be converted into more active derivatives (Massoud and Sharifan, 2020). Based on the results, the number of *L. acidophilus* bacteria under NC stress (NaCl 2 %) treatment at 21st day of storage period (5.83 ± 0 log CFU/mL) was higher than that of 28 days of storage period (5 logs CFU/mL) (Gandhi and Shah, 2015). They found that the *L. acidophilus* bacteria viability decreased parallel to increasing salt concentration from zero to 5% and storage period from 1 h to 1 week. Therefore, BEO treatment is considered a suitable environment for *L. acidophilus* survival, and it is also recommended for consumers.

Titratable acidity and pH

The pH and TA content of yogurt were considerably affected during the storage period ($P < 0.01$) (Figures 2 and 3).

Parallel to the decrease in pH value, TA was considerably increased. Control and BEO samples had the lowest content of pH in comparison to other treatments. In terms of pH and TA values, there were no considerable differences between control and BEO treatments during the 28 days storage period ($P > 0.01$) (Figures 3 and 4).

In addition, there were no considerable differences between treatments under stress with NC and BS exclusively after 28 days of storage ($P > 0.01$). BEONCBS treatment had the most TA and less pH among all samples, and then NCBS treatment had the lowest and the most pH parameter and TA during the storage period. At the end of the 28th day, the control treatment had the highest pH (4.37 ± 0.01) and the lowest TA (1.32 ± 0.02), whereas the treatment under stress with BEONCBS had the lowest pH (3.90 ± 0.03) and the highest TA (1.43 ± 0.01). As mentioned above, the total pH decreased during refrigeration at 4°C for all the treatments ($P < 0.01$). The range of pH of all the samples varied from 3.9 to 4.4 during the period of storage. The results showed that the control treatment had more pH and less acidity than others ($P < 0.01$). Among the stress treatments, BEO treatment had a more pH and less acidity than others. The main reason for TA and pH changes could be attributed to the lactose alteration to lactic acid during fermentation, starter culture type, storage duration, and incubation temperature (Singh *et al.*, 2011). Catabolism of lactose by the starter bacteria resulted in lactic acid production. For this reason, pH decreased, and the acidity of samples gradually increased during the storage period (Ramasubramanian *et al.*, 2008). *L. acidophilus* reduced pH values and increased acidity of yogurt by producing lactic acid (Mortazavian *et al.*, 2006). In stress treatments with NC and BS, pH values decreased, and acidity values increased during storage. Salt impressed the

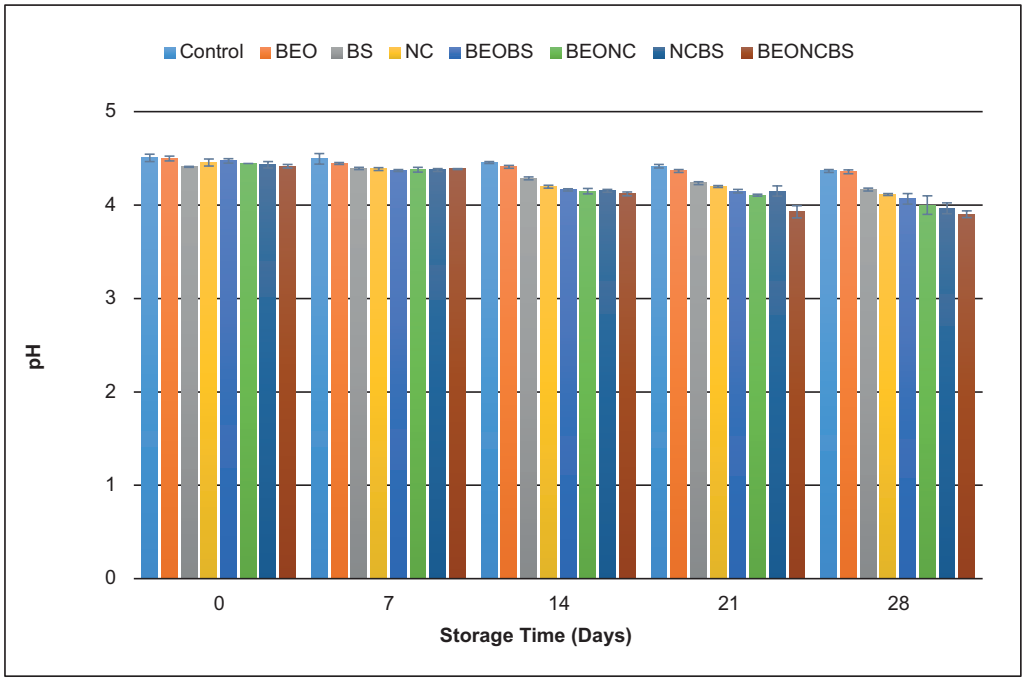

Figure 3. Effect of *Bunium persicum* essential oil (BEO), NaCl (NC), bile salts (BS), and their combinations on changes to the pH of probiotic yogurt. Deviation bars designate the standard error of the method (n = 3).

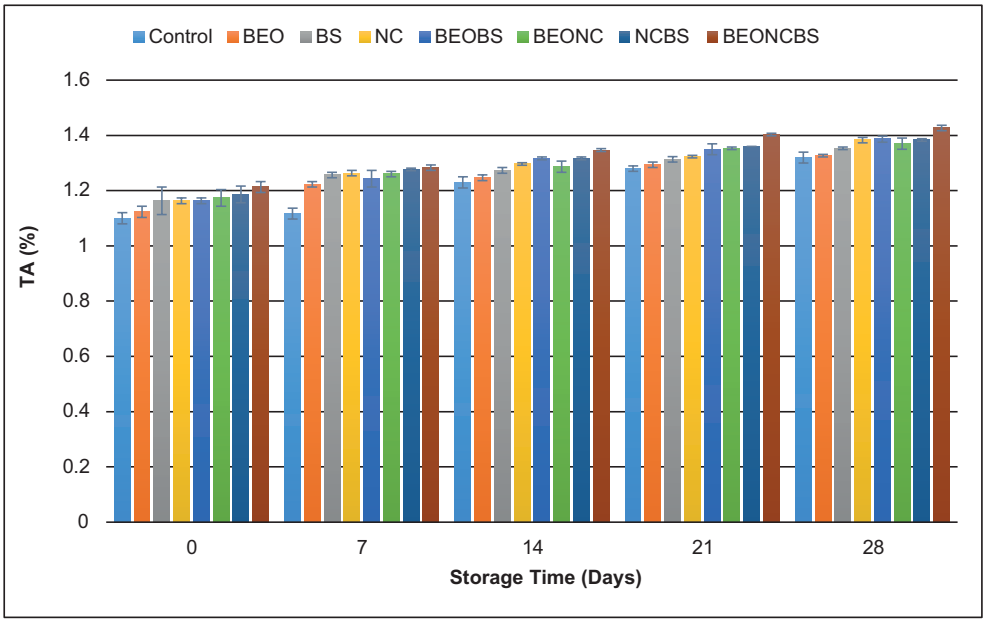

Figure 4. Effects of *Bunium persicum* essential oil (BEO), NaCl (NC), bile salts (BS), and their combinations on changes to the acidity of probiotic yogurt. Deviation bars designate the standard error of the method (n = 3).

growth of starter culture and consequently changed the production of lactic acid content (Gandhi and Shah, 2015). Moreover, lactic acid production correlated to the viable counts of probiotic strain and prevented starter cultures' growth due to acid production during the storage (Ahari *et al*., 2020).

Syneresis

The percentage of syneresis of all samples was considerably increased during storage ($P < 0.01$). The amount of syneresis was higher for stress treatments than the control sample (except for the BEO treatment) (Figure 5).

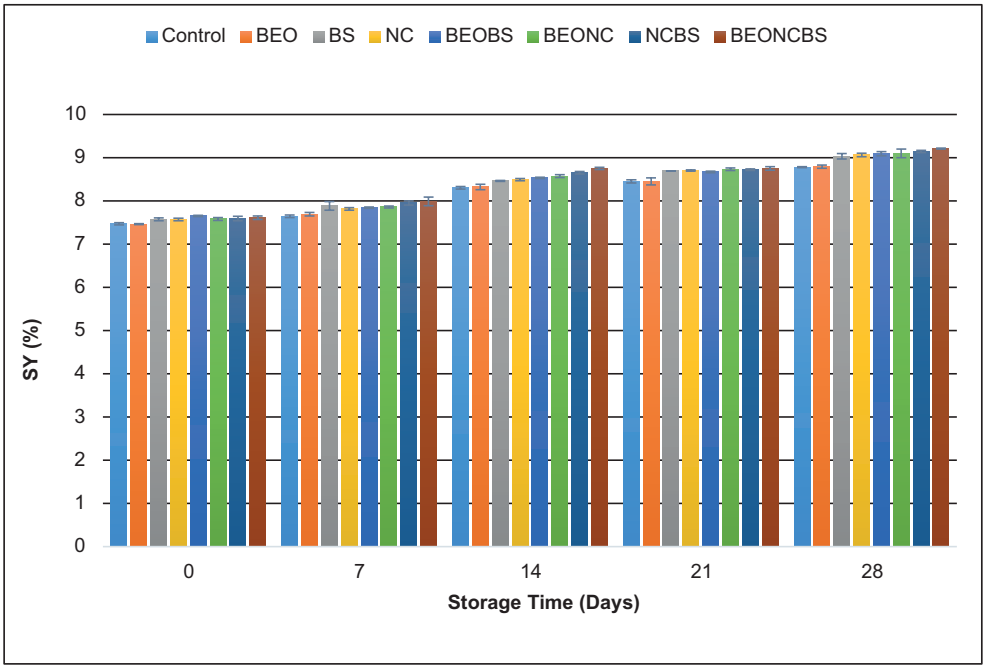

Figure 5. Effects of *Bunium persicum* essential oil (BEO), NaCl (NC), bile salts (BS), and their combinations on changes to the syneresis of probiotic yogurt. Deviation bars designate the standard error of the method (n = 3).

Because proteins were hydrolyzed during the storage period, free amino acids and short-chain polypeptides were released. These compounds were hydrophilic and increased water-holding capacity (Cumby *et al*., 2008). Protein denaturation may also have a different impact on the capacity of water holding and syneresis content (Isleten and Karagul-Yuceer, 2006). The control and BEO treatments had lower syneresis than the other treatments ($P < 0.01$). Lower pH could be an important impact on forming the gel network, and the gel network was weak for treatments with higher pH because of higher electrostatic repulsion among micellar caseins. This might cause a weak gel network during incubation and result in higher syneresis in yogurt (Yangilar and Yildiz, 2018). Hence, control and BEO treatments were expected to have a lower syneresis than the other stress treatments. In addition, probiotic strain and starter culture bacteria hydrolyzed proteins and caused higher syneresis in yogurt during storage in the refrigerator. Syneresis influenced the texture of yogurt and caused weak network gel (Seitz, 1990).

Sensory properties

The addition of *B. persicum* essential oil (BEO) changed the sensory properties (flavor, texture, and general acceptance) of probiotic yogurt considerably ($P < 0.01$). Table 2 showed texture, flavor, and overall acceptance scores of probiotic yogurts decreased during all samples' storage period. On the first day of the storage period, no considerable differences were observed in all samples ($P > 0.01$).

On the 28th day of storage, control treatment and the stress treatment with BEONCBS had the highest and the lowest scores in all sensory properties, respectively. In addition, there was no considerable difference between treatments under stress containing NC and BS

Table 2. Effects of *Bunium persicum* (Boiss) essential oil (CEO), NaCl (NC), bile salts (BS), and their combinations on the organoleptic properties/scores* of probiotic yogurts.**

Sensory property	Yogurt sample	Storage time (day)				
		0	7	14	21	28
Flavor	Control	5 ± 0^{a}	5 ± 0^{a}	5 ± 0^{a}	4.1 ± 0.57^{b}	3.8 ± 0.42^{bc}
	BEO	5 ± 0^{a}	5 ± 0^{a}	4.9 ± 0.32^{a}	4 ± 0.47^{bc}	3.7 ± 0.48^{c}
	BS	5 ± 0^{a}	5 ± 0^{a}	4 ± 0^{bc}	3.9 ± 0.32^{bc}	3.3 ± 0.48^{d}
	NC	5 ± 0^{a}	5 ± 0^{a}	4 ± 0^{bc}	3.9 ± 0.32^{bc}	3.3 ± 0.48^{d}
	BEOBS	5 ± 0^{a}	5 ± 0^{a}	4 ± 0^{bc}	3.9 ± 0.32^{bc}	3.3 ± 0.48^{d}
	BEONC	5 ± 0^{a}	5 ± 0^{a}	4 ± 0^{bc}	3.9 ± 0.32^{bc}	3.3 ± 0.48^{d}
	NCBS	5 ± 0^{a}	5 ± 0^{a}	4 ± 0^{bc}	3.3 ± 0.48^{d}	3.2 ± 0.42^{d}
	BEONSBS	5 ± 0^{a}	5 ± 0^{a}	4 ± 0^{bc}	3 ± 0^{d}	3 ± 0^{d}
Texture	Control	5 ± 0^{a}	5 ± 0^{a}	4.4 ± 0.52^{b}	4.3 ± 0.48^{b}	3.4 ± 0.52^{d}
	BEO	5 ± 0^{a}	5 ± 0^{a}	4.3 ± 0.48^{b}	4.2 ± 0.42^{bc}	3.3 ± 0.48^{de}
	BS	5 ± 0^{a}	5 ± 0^{a}	4 ± 0^{c}	3.7 ± 0.48^{c}	3.3 ± 0.48^{de}
	NC	5 ± 0^{a}	5 ± 0^{a}	4 ± 0^{c}	3.7 ± 0.48^{c}	3.3 ± 0.48^{de}
	BEOBS	5 ± 0^{a}	5 ± 0^{a}	4 ± 0^{c}	3.7 ± 0.48^{c}	3.3 ± 0.48^{de}
	BEONC	5 ± 0^{a}	5 ± 0^{a}	4 ± 0^{c}	3.7 ± 0.48^{c}	3.3 ± 0.48^{de}
	NCBS	5 ± 0^{a}	5 ± 0^{a}	4 ± 0^{c}	3 ± 0^{e}	3 ± 0^{e}
	BEONSBS	5 ± 0^{a}	5 ± 0^{a}	4 ± 0^{c}	3 ± 0^{e}	3 ± 0^{e}
Overall acceptability	Control	5 ± 0^{a}	5 ± 0^{a}	4.3 ± 0.48^{b}	4.2 ± 0.63^{bc}	3.9 ± 0.32^{bcdef}
	BEO	5 ± 0^{a}	5 ± 0^{a}	4.3 ± 0.48^{b}	4.1 ± 0.57^{bcd}	3.8 ± 0.42^{cdefg}
	BS	5 ± 0^{a}	4 ± 0^{bcde}	3.7 ± 0.48^{defg}	3.7 ± 0.48^{defg}	3.5 ± 0.53^{fg}
	NC	5 ± 0^{a}	4 ± 0^{bcde}	3.7 ± 0.48^{defg}	3.7 ± 0.48^{defg}	3.5 ± 0.53^{fg}
	BEOBS	5 ± 0^{a}	4 ± 0^{bcde}	3.7 ± 0.48^{defg}	3.6 ± 0.52^{efg}	3.5 ± 0.53^{fg}
	BEONC	5 ± 0^{a}	4 ± 0^{bcde}	3.7 ± 0.48^{defg}	3.7 ± 0.48^{defg}	3.5 ± 0.53^{fg}
	NCBS	5 ± 0^{a}	4 ± 0^{bcde}	3.8 ± 0.42^{cdefg}	3.7 ± 0.48^{defg}	3.5 ± 0.53^{fg}
	BEONSBS	5 ± 0^{a}	4 ± 0^{bcde}	3.8 ± 0.42^{cdefg}	3.4 ± 0.52^{g}	3.4 ± 0.52^{g}

*Mean ± standard deviation.
**Each column and row with the same letters indicates that there was no considerable difference at $P > 0.01$.

individually and combined with CBEO in each specified day of storage period (P > 0.01). Probiotic strains inhibited reactions related to producing flavor and odor, and they were very complex, and it is necessary to perform sensory evaluations to specify the suitable sample. The scores for the flavor of probiotic yogurt decreased during the storage period; however, the control sample had a high score among all treatments. The scores for control and stress treatments with BEONCBS were 3.80 ± 0.42 and 3 ± 0, respectively. The decrease of flavor score might have been due to the rising acidity value and diminishing activity of flavor-producing bacteria (Donkor *et al*., 2006). Proteolytic *Lactobacillus* strains, such as *L. acidophilus*, produced effective flavor ingredients via carbohydrate metabolism, proteolysis, and mild lipolysis. These enzymes hydrolyze casein and produce large and medium peptides. Peptides might be decomposed into small peptides and free amino acids by proteolytic enzymes produced from microflora of starter bacteria, nonacidic lactic acid, and probiotics. These compounds are responsible for flavor in dairy products (Seitz, 1990). The texture score of the probiotic yogurt also decreased during the storage period. The highest score for texture was attributed to the control sample (3.40 ± 0.52) up to the end of the storage period. The percentage of syneresis is related to the firmness of texture. The more syneresis during storage, the looser the texture results in yogurt (Ayar and Gurlin, 2014). The overall acceptance score decreased with time in all samples due to attention to flavor and texture scores. At the end of the storage period, the control sample had the highest score (3.90 ± 0.32), and the stressed sample with BEONSBS had the lowest score (3.40 ± 0.52) with due attention of overall acceptance.

Conclusion

The number of *L. acidophilus* and organoleptic acceptability decreased; however, the percentages of titratable acidity and syneresis increased during the storage period for all samples. Use of *B. persicum* Boiss essential oil individually or combined with NaCl and bile salts at 50% MIC in yogurt, which was kept in a refrigerator for 28 days of the storage period, provided a suitable environment for bioavailability of *L. acidophilus*. Herbal essential oils are being used as a new strategy for the viability increment of probiotic strains in fermented probiotics such as yogurt, in addition to meeting consumer expectations regarding the properties of the product sensory BEO could be improved the nutritional, medicinal, and sensory properties and maintenance of yogurt. However, the MIC of plant essential oils against probiotics is high, whereas at much lower concentrations, the essential oils have inhibitory effects on pathogens, so an important impact of this article is increment viability of bacteria in probiotic products. Therefore, the use of essential oils in low doses can kill pathogens without harming the probiotics, and it would be suggested that evaluate the texture properties of yogurt in storage time in a future article.

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