

Probiotics survival, antioxidant activity and sensory properties of yogurt flavored with herbal essential oils

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Abstract

The objective of this study was to investigate the viability of the probiotics, antioxidant activity and organoleptic acceptability of probiotic-yogurt containing essential oils (peppermint, basil and zataria). Samples were produced using probiotic organisms *Lactobacillus acidophilus* LA5, *Lactobacillus fermentum* and *Bifidobacterium* Bb-12 besides the starter culture and added essential oils. The presence of essential oils did not affect LA5 population in yogurt during 4 weeks of storage but the growth of Bb12 was retarded. Water extract of zataria-yogurt showed the highest inhibitory effect on DPPH radicals followed by peppermint and basil samples. Peppermint yogurt received the highest score followed by basil and control yogurt but zataria sample did not reach the consumer acceptability limit (score >5). The present findings suggest that adding zataria, basil or peppermint essential oils into probiotic yogurt formulation improves the antioxidant potential of the product. Peppermint and basil samples showed both good antiradical activity and sensory acceptability.

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Keywords

Antioxidant activity
Essential oil
Probiotics
Sensory properties
Yogurt

Introduction

Lactic acid bacteria (LAB) play an important role in determining the positive health effects of fermented milks and related products and yoghurt is redefined as a probiotic carrier food (Shiby and Mishra, 2013). Probiotics which are live beneficial microorganisms, when administered in appropriate amounts, also have been shown to have various health benefits. More than 90% of probiotic products contain species of lactobacilli and bifidobacteria (Shah, 2000).

Herbal products (spices, essential oils and extracts) have been used as a source of functional flavouring agents. There are several published works about the health benefits of herbs including antimicrobial, antioxidant, anti-inflammatory and anticarcinogenic properties (Ozcan and Ahgul, 1995; Erkmen and Özcan, 2001; Srinivasan, 2004; Tapsell *et al.*, 2006; Ozcan *et al.*, 2007; Ozcan, 2009). Combinations of probiotics with herbs may provide further antimicrobial-therapeutic properties. However as herbs are antimicrobials, they may affect viability of probiotic microorganisms. *In-vitro* studies that tested herbs on the growth of selected probiotics showed that herbal products significantly enhanced the growth of probiotics while inhibiting pathogens (Sutherland *et al.*, 2009; Be *et al.*, 2010).

Therefore, the objective of this study was to (I) produce probiotic-yogurt containing herbal essential oils (pepper mint, basil and zataria), (II) investigate the effect of these essential oils (EOs) on the viability of the chosen probiotics in yogurt samples and (III) investigate the antioxidant activity and sensory properties of the produced yogurts.

Materials and Methods

Essential oils and chemicals

The EOs (*Mentha piperita* (peppermint), *Ocimum basilicum* (basil) and *Zataria multiflora* Boiss.) were obtained from Barij company, Kashan, Iran. The EOs were stored in airtight dark glass vials at 4°C. All culture media and chemicals used were of analytical grade or the highest grade available and were obtained either from Sigma-Aldrich or Merck (Germany).

Gas chromatography and gas chromatography-mass spectrometry

EOs were analyzed by gas chromatography (GC) (Thermo Quest® 2000, UK). The chromatograph was equipped with a DB5 capillary column (Aligent Technologies, USA) (30 × 0.25 mm ID × 0.25 µm film thickness) and the data were acquired under

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the following conditions: initial temperature 50°C; rate of increase of temperature 2.5°C per minute, final temperature 265°C and injector temperature 250°C. An injection volume of 0.5 µL was employed using the autosampler (autosampler 7693 – 100 positions, Agilent Technologies, USA). The carrier gas was helium and the split ratio was 120. The column head pressure was 24.9 kPa. An Agilent 6890 Flame Ionization Detector (Agilent Technologies, USA), operated at 200 Hz, was used. EOs were also analyzed by gas chromatography mass spectroscopy (GC/MS) (Thermo Quest Finnigan®, UK) using the same capillary column and analytical conditions indicated above. The MS was run in the electron ionization mode using an ionization energy of 70 eV. Components were identified based on the comparison of their relative retention times and mass spectra with those of standards (Adams, 2007). Alkanes were used as reference points in the calculation of relative retention indices (RRI).

Probiotics and starter culture

The probiotic organisms *Lactobacillus acidophilus* LA5, *Lactobacillus fermentum* and *Bifidobacterium* Bb-12, were selected for this study based on the previous studies (Behrad *et al.*, 2009; Be *et al.*, 2010). Freeze-dried starter culture (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) and probiotics were obtained from Chr. Hansen, Ltd, Denmark.

Preparation of probiotic yogurt with essential oils

According to the manufacturer's instruction on yogurt preparation, 2 L of milk (local supplier), with the required fat content (3.9 g/100 ml) was mixed vigorously with 2% of skimmed milk powder (Pajan Dairy Co., Iran) and 5% of sucrose (Sigma-Aldrich, Germany) to ensure an efficient mixing. Then, remaining 2 L of milk was added and mixed thoroughly. The mix was heated to 85°C for heat treatment for 30 min, then cooled to 43-45°C in a water bath while stirring slowly with a hand stirrer and inoculated with the prepared yogurt starter culture (0.02% v/v). The mix was aliquoted into four equal portions (1 L each). Three portions were added with either one of the EOs (0.5 % v/v) and one portion used without EOs as control-probiotic yogurt. Each portion was inoculated with probiotics LA5 (0.02% w/v), *L. fermentum* (0.02% w/v) and Bb12 (0.02% w/v). The inoculated mixtures were aliquoted into cups and incubated at 40°C for fermentation and terminated when pH reached 4.5 (approximately 6 h), then yogurt cups were cooled rapidly in a refrigerator at 4±1°C for storage.

Determination of pH

The pH of yogurts was determined every hour at 20±1°C during fermentation and also storage at 4°C. Yogurt sample (1g) was mixed with distilled water (1:1), and the pH was measured using a pH meter (Jenway, UK), calibrated routinely with fresh pH 4.01 and 6.86 standard buffers (Institute of Standards and Industrial Research of Iran, 2006).

Enumeration of probiotic bacteria

Clindamycin was used in order to inhibit the growth of lactic acid bacteria in the starter culture but allow growth of LA5. MRS agar was prepared with addition of 0.05% of clindamycin stock solution per L of medium. Enumeration of total lactobacilli was carried out using MRS agar without clindamycin. Enumeration was conducted by aseptically mixing 1ml of yogurt sample with 9 ml of buffered peptone water. The sample was thoroughly mixed and serial dilutions were performed using peptone water as the diluent. Empty petri dishes were inoculated with 1ml of diluted yogurt, followed by the addition of 15 ml melted MRS agar (45°C). The petri dishes were covered and the contents mixed thoroughly by gentle tilting and swirling. The petri dishes were inverted and incubated anaerobically in jars containing anaerogen sachets at 37°C for 24-48 hours to determine viable count on days 0, 7, 14, 21 and 28 (Vijayalakshmi *et al.*, 2014).

To inhibit starter culture, but allow growth of Bb12, the combination of dicloxacillin (10%), lithium chloride (11%) and cysteine hydrochloride (10%) was used (Chr. Hansen, 2007). The Bb12 viable cell counts were carried out by plating diluted yogurt samples using the MRS agar with addition of 0.5% of dicloxacillin stock solution, 1% of lithium chloride stock solution and 0.5% of cysteine hydrochloride stock solution per L of medium according to the pour plate method. Plates were incubated anaerobically in jars containing anaerogen sachets at 37°C for 48 hours to determine viable count on days 0, 7, 14, 21 and 28.

Antioxidant activity

1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay was applied to evaluate antioxidant activity. Antioxidant potential of yogurt samples during storage for 0, 7, 14, 21 and 28 days at 4°C were determined with a stable radical substrate, DPPH as described by Vijayalakshmi *et al.* (2014). Briefly, yogurt sample (1 g) was mixed with distilled water (1:1) and centrifuged at 10,000 rpm for 30 min. To 3 ml of 60 mM DPPH in ethanol, 250 µl of each yogurt extracts was added. The mixture was shaken vigorously.

Prepared samples were aliquoted into microplate wells and incubated in the dark. After 20 min, the absorbance was read at 517 nm and the results were compared with the control, which contained 250 μ l of dH₂O instead of the water extracts of probiotic-yoghurts with or without EOs. The percentage of antioxidant activity was calculated as percentage inhibition of DPPH radical formation using the formula (1) as below:

$$\text{Percentage of inhibition} = \left(\frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} \right) \times 100 \quad (1)$$

Sensory evaluation

The sensory evaluation was conducted through consumer taste panels using nine point hedonic scale (Gonzalez *et al.*, 2011) by procedures discussed in Moskowitz *et al.* (2006). For consumer acceptance sensory tests, a minimum of fifty ratings per product were considered desirable for the precision of the statistical analysis (Moskowitz *et al.*, 2006). Thus 30 participants between the ages of 18-25 (22 females and 8 males) from Amol University of Modern Special Sciences, Amol, were recruited to take part in consumer taste panel. Consumer acceptance of the experimental products was evaluated using a hedonic scale of 1-9 where 1 corresponds with “dislike extremely” and 9 corresponds “like extremely” in two consumer panels.

Statistical analysis

All data analyses were performed using the SPSS software version 22.0. One-way ANOVA was applied to evaluate the difference between the yogurts with respect to the sensory attributes. In order to compare the mean value between samples Least Significant Difference (LSD) was used (Gonzalez *et al.*, 2011) and data was significant if the p value was found to be < 0.05 . Results were presented as the mean \pm standard deviation. One way ANOVA with post-hoc mean separation using LSD was carried out for statistical analysis of the treatment and storage effect on the viable microorganisms (Hemsworth *et al.*, 2011) and pH and antioxidant activity (Ali, 2010) over the 28 day period of storage. Results were presented as the mean and the standard error of the mean (\pm SE). Data was significant if the p value was found to be < 0.05 .

Results

GC-MS analysis resulted in the identification of 25 components for peppermint EO, 23 components for basil EO and 12 components for that of zataria, representing more than $> 91\%$ (v/v) of the oils in

Table 1. The main components of peppermint, basil and zataria EOs

EOs' component	Amount (%)
Peppermint	
menthol	32.92
menthone	31.11
menthyl acetate	6.58
1,8 cineole	3.98
Basil	
linalool	58.63
cadinol	10.01
α -bergamotene	7.62
γ -cadinen	4.92
Zataria	
carvacrol	71.12
gamma-terpinene	7.34
alpha-pinene	4.26
eucaliptol	3.37

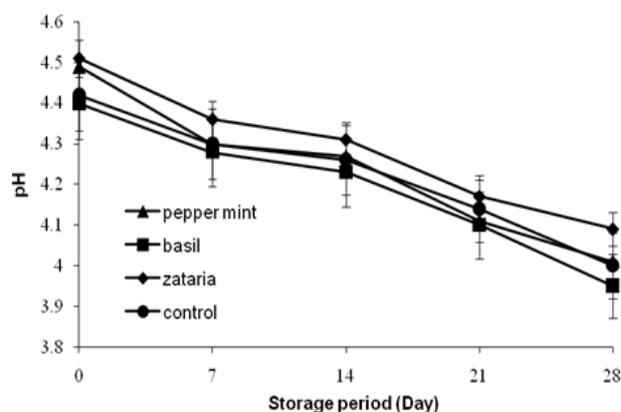


Figure 1. Changes in pH of control and EO treated yogurts during refrigerated ($4\pm 1^\circ\text{C}$) storage.

each case. The main components of EOs are shown in Table 1. The pH for control yogurt (plain) was approximately the same as pH of EO treated yogurts. An overall decline of pH of yogurts occurred during refrigerated storage. The pH for all yogurts reduced ($p < 0.05$) from control yogurt except for zataria treated samples (Figure 1).

The results of LA5 and total lactobacilli enumeration are shown in Figure 2. The presence of essential oils such as basil or peppermint do not affect the viable count of LA5 or total lactobacilli on day 0 compared to control probiotic-yogurt. But the presence of zataria resulted in a lower count of viable lactobacilli and also LA5 in zataria treated yogurt ($P < 0.05$) on day 0 of storage compared to control and other two EO treated yogurts. The viable count of LA5 and lactobacilli increased from day 0 to day 7 of storage for all yogurts but with reduction in zataria treated sample ($P < 0.05$). The viable LA5 and lactobacilli count however reduced from day 7 to day 28 of storage for all yogurt samples with the fastest rate occurring in control-yogurt. The viable LA5 count on day 28 of storage for basil treated yogurt (4.8×10^6 CFU/ml) was not significantly different from peppermint treated yogurt ($P > 0.05$).

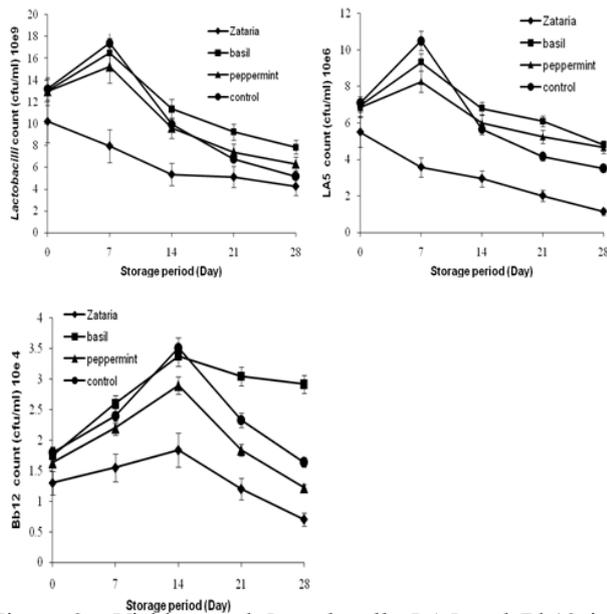


Figure 2. Viable total *Lactobacilli*, LA5 and Bb12 in essential oil treated yogurts during refrigerated storage.

Similar to the results of total lactobacilli and LA5 counts in EO- treated yogurts (Figure 2), the presence of basil and peppermint EOs did not affect the viable count of Bb12 on day 0 compared to control-yogurt. But the presence of zataria EO resulted in a lower Bb12 count on the same day of storage compared to all the other yogurts ($P < 0.01$). The viable count of Bb12 increased from day 0 to day 14 in all yogurts. But from day 14 to day 28 of storage, all samples had a reduced viable count with the slowest rate occurring in basil treated yogurt ($P < 0.05$). On day 28, the viable count of zataria treated yogurt (0.7×10^4 CFU/ml) was significantly different from control yogurt and other treated samples. The basil yogurt resulted in significantly ($P < 0.05$) higher viable count (2.92×10^4 CFU/ml) compared to control yogurt (1.64×10^4 CFU/ml).

The results of antioxidant assay are shown in Figure 3. The addition of EOs, seemed to increase the antioxidant potential of yogurts compared to control yogurt at all storage periods (Figure 3). The highest antioxidant activity was obtained on day 7 for zataria treated yogurt (76.5%) followed by peppermint (54.2%), basil treated yogurt (45.2%) and control-yogurt (31.3%). At the end of storage period (day 28) the highest antioxidant activity was recorded for zataria yogurt (18.9%) followed by peppermint yogurt (14.2%) and basil (10.3%). As seen in Figure 3, zataria treated yogurt showed the highest antioxidant activity at all storage periods compared to other EO treated and control yogurts ($P < 0.05$). Control yogurt showed the lowest antioxidant activity significantly ($P < 0.05$) different from EO

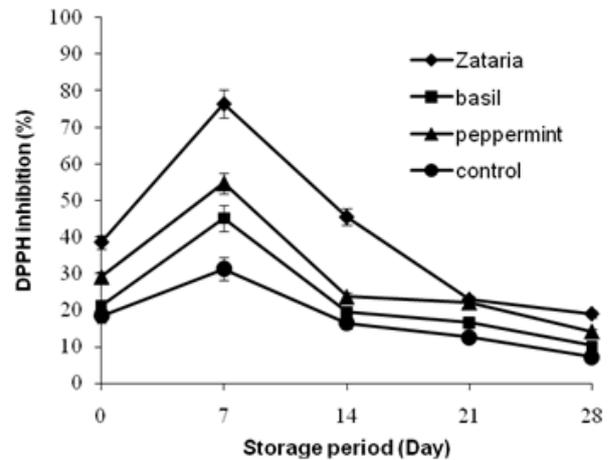


Figure 3. DPPH inhibition by water extract of essential oil treated and control yogurts during refrigerated storage.

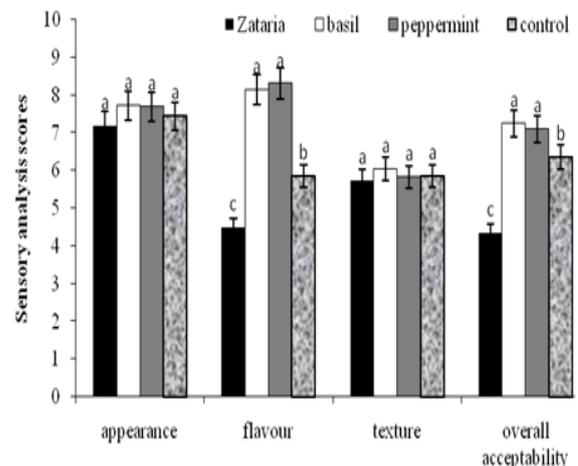


Figure 4. The scores for the sensory analysis of probiotic-yogurts containing different essential oils.

treated yogurt samples.

In the sensory tests yogurt samples were evaluated for appearance, flavour, texture and overall acceptability. The results of the sensory evaluation are shown in Figure 4. The mean scores for the appearance of basil and peppermint treated yogurt were higher than the control-yogurt. Figure 4 indicates that the mean scores for the appearance of probiotic yogurt with basil and peppermint were within the acceptable range, but there was no significant difference between the types of yogurt ($P > 0.05$).

The results in Figure 4 show that there was a statistically significant difference between the mean scores for the flavour of both basil and peppermint probiotic yogurts with zataria and control yogurt ($P < 0.05$). Also, as it is obvious from the results control probiotic-yogurt received higher flavor scores than zataria treated yogurts ($P < 0.05$). The sensory analysis data indicate that the texture scores of EO treated probiotic yogurts were not significantly different from control yogurt ($P > 0.05$). In case of

overall acceptability, peppermint and basil treated yogurts were significantly different ($P < 0.05$) from the other yogurts. The mean scores for zataria yogurt were significantly lower than the control ($P < 0.05$).

Discussion

In the present study, using of herbal essential oils (peppermint, basil and zataria) and probiotic strains (*Lactobacillus acidophilus* LA5, *Lactobacillus fermentum* and *Bifidobacterium* Bb-12) in yogurt with the aim of evaluating the antioxidant activity and sensory properties of the products was developed. Probiotic-yogurts with various flavours such as cardamom, cinnamon, nutmeg, peach, blackberry and strawberry have been developed by some researchers (Cruz *et al.*, 2010; Gonzalez *et al.*, 2011; Vijayalakshmi *et al.*, 2014). Availability and cost effective nature of herbal essential oils make them suitable food additives for enhancing the functionality of commercial yogurts.

As seen in the results, overall decline of pH of yogurts occurred during refrigerated storage. The pH for all samples reduced from the initial values of approximately 4.45 to between 3.95 and 4.09 by day 28 of storage ($P < 0.05$). The presence of herbs did not make the pH of herbal-yogurts any different ($P > 0.05$) from control yogurt, it indicates that the bacteria continuo fermentation and acid production. Our results are in accordance with the findings of Behrad *et al.* (2009), who showed that pH for control yogurt was almost the same as pH of spice treated yogurts.

The survival of probiotic bacteria in yogurt or similar foods during refrigerated storage until consumption is a crucial factor in the field of probiotic products. According to several studies in this regard, to achieve optimal potential beneficial therapeutic effects, the number of probiotics in a product at the time of consumption should at least meet a suggested "therapeutic minimum" 10^5 - 10^6 CFU/g or ml of the final product for functionality and 10^8 CFU/g for presentation to the gut for any functional benefit (Granato *et al.*, 2010; Cruz, Cadena, Faria *et al.*, 2012; Cruz, Castro, Faria *et al.*, 2012).

Although the same volume of LA5 and Bb12 was added at the time of yogurt production, viable colonies for the Bb12 at the first day of storage (day 0) was quite different but for LA5 and total lactobacilli a high viable count was maintained. The results for LA5 and lactobacilli are comparable to the levels of other commercial probiotics such as *L. delbrueckii* ssp. *bulgaricus* with levels of 10^5 CFU/ml in probiotic stirred-type yogurt (Marafon *et al.*, 2011).

This may indicate freeze drying may have killed more Bb12 culture than LA5 and other lactobacilli prior to manufacture of the yogurt (Vijayalakshmi *et al.*, 2014). In the present study, after 4 weeks of storage, all the EO treated probiotic yogurts contained an acceptable level of LA5 (10^6 CFU/ml) and total lactobacilli (10^9 CFU/ml). These results are in accordance with Behrad *et al.* (2009) that concluded LA5 were maintained in spice (cinnamon and liquorice) yogurt for 28 days storage at the level between 10^6 - 10^7 CFU/ml but in regard to Bb12 our results were in contrast to theirs. A number of studies have showed that the survival of probiotic bifidobacteria is often low in yogurt (Shah, 2000; Lourens-Hattingh and Viljoen, 2001; Gonzalez *et al.*, 2011), as seen in the present study.

In this study the viable numbers of probiotics were lower when zataria essential oil was used. This may be due to the main component of zataria essential oil (carvacrol $> 70\%$) that has a great inhibitory effect on Gram-positive bacteria and is considered as a strong antimicrobial (Zomorodian *et al.*, 2011). The increase in viable count of probiotics during the first 7 days coincided with the significant reduction in pH observed in all the samples. In a similar way, the sharp increase in viable count of Bb12 from day 7 to day 14 coincided with the approximately stable pH recorded (slight change in pH) during this period in all samples. These results indicate that, the reduction in viable count which occurred in consistent manner in all yogurts may be attributed to the organic acid accumulation as the results of growth and fermentation. Altogether the conversion of lactose to lactic acid, the composition of bacterial starter culture, and duration of storage and fermentation temperature could be the reason for the decrease in pH during storage (Singh *et al.*, 2011).

As indicated in Figure 3, probiotic yogurt containing zataria EO showed the highest antioxidant activity at all storage periods compared to other EO and control yogurts ($P < 0.05$). Our findings are in accordance with the results of Amirdivani and Baba (2011) who observed that highest DPPH inhibition of herbal yogurts (samples treated with peppermint, dill and basil) occurred after 7 days of refrigeration and the antioxidant activity decreased toward the end of the storage period, it may be due to the gradual release of EOs' antioxidant components at first 7 days and then the decrease of the these compounds to the end of the storage period. Also, Behrad *et al.* (2009) who studied the effects of manipulation of probiotics fermentation of yogurt by cinnamon and licorice on yogurt properties found that the highest antioxidant activity of spice added yogurts occurred on day 7 of

storage. In a study conducted by Vijayalakshmi *et al.* (2014) probiotic yogurts containing spice oleoresins (cardamom, cinnamon and nutmeg) with acceptable sensory properties were produced. In contrast to our findings, they observed that the highest antioxidant activity of spice added yogurts occurred on day 1 of storage.

In the sensory tests yogurt samples were evaluated for appearance, flavour, texture and overall acceptability. As it is concluded from the results of flavour and overall acceptability analysis, zataria yogurt did not reach the consumer acceptability limit (score >5) but peppermint, basil and control samples were well accepted by the consumers; there was not significant difference between basil and peppermint probiotic-yogurts. In regard with appearance and texture, all yoghurt samples obtained scores above 5 in the nine point hedonic scale, which implied that essential oils did not affect the textural properties of the yogurt products. In the present study, overall yogurt containing, peppermint and basil were highly accepted by consumers with higher preference towards peppermint and zataria-yogurt scored less in the products.

However, the results of this study suggests the possibility of producing commercially acceptable products containing peppermint, basil and lower concentration of zataria EO when compared with a recent study conducted on consumer acceptability with commercially available yogurt products (Cruz, Cadena, Faria *et al.*, 2012; Cruz, Castro, Faria *et al.*, 2012).

Conclusion

In conclusion, probiotic yogurt containing strains of *L. fermentum*, LA5 and Bb12 and essential oils of peppermint, basil and zataria was successfully manufactured with viable probiotic counts up to the acceptable range and good antioxidant activity during 28 days. It is worth to note that the survival of lactobacilli in all yogurt samples was higher than *bifidobacterium*. Also, both control (plain probiotic sample) and treated yogurt (contained probiotics and essential oils) showed considerable inhibitory effect on DPPH radicals. The effectiveness of these herbal-yogurts to halt free radicals needs to be further investigated under in vivo condition.

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