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Changes in yogurt fermentation characteristics, and antioxidant potential and *in vitro* inhibition of angiotensin-1 converting enzyme upon the inclusion of peppermint, dill and basil

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ABSTRACT

The present study investigated the effects of peppermint (*Mentha piperita*), dill (*Anethum graveolens*) and basil (*Ocimum basilicum*) on yogurt formation, proteolysis and inhibition of angiotensin-1 converting enzyme (ACE). Herbal-yogurts had faster rates of pH reduction than plain-yogurt. All herbal-yogurts had higher ($p < 0.05$) antioxidant activities than plain-yogurt, both at the end of fermentation and throughout the storage period. The o-phthalaldehyde (OPA) peptides in herbal-yogurts increased by 28–36% after 7 days of storage. All herbal-yogurts showed higher anti-ACE activity than plain-yogurt at corresponding storage periods. *M. piperita* yogurt had highest inhibitory effect on ACE activity throughout the storage period. Peppermint, dill and basil may be used to modify microbial fermentation of milk with the intention of producing dairy products with higher antioxidant and enhanced anti-ACE activities.

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1. Introduction

Yogurt is a widely consumed as functional food due to its good taste and nutritional properties (rich in potassium, calcium, protein and vitamin B) and excellent vehicle to deliver probiotics to consumers (Reid et al., 2003). Regular consumption of yogurt is thought to be beneficial in the strengthening of the immune system, improvement in lactose digestion, blood glucose management (Yadav, Jain, & Sinha, 2007) and the reduction of constipation, diarrhea, colon cancer, inflammatory bowel disease and allergies (Adolfsson, Meydani, & Russel, 2004).

The beneficial health effects of yogurt have partly linked to the proteolysis products, produced during fermentation and storage in particular, a group of peptides can lower the blood pressure in hypertensive patients (FitzGerald, Murray, & Walsh, 2004). An important intermediary factor for controlling hypertension is the action of the angiotensin-I converting enzyme (ACE-I) (Hernandez-Ledesma, Martin-Alvarez, & Pueyo, 2003). ACE-I hydrolyzes the decapeptide angiotensin-I to yield the octapeptide angiotensin-II (Skeggs, Khan, & Shumway, 1956). Angiotensin-II is both a potent vasoconstrictor and stimulator for the synthesis and release of aldosterone which subsequently increases blood pressure by

promoting sodium retention in the distal tubules (Lieberman, 1975). Hence, the inhibition of ACE-I is considered a useful therapeutic approach in the treatment of high blood pressure (Johnston & Franz, 1992).

Cardiovascular disease is a stress-related chronic disease (Serdula, Byers, Simoes, Mendlein, & Coates, 1996) and thus the consumption of adequate antioxidants forms an important strategy to manage further degenerative effects in these diseases (Thompson & Godin, 1995). Phenolic phytochemicals are secondary metabolites of plant origin that constitute an important part of both human and animal diets (Shetty, Clydesdale, & Vatter, 2005). Recent studies have shown that phenolic phytochemicals have high antioxidant activity and certain therapeutic properties (Shetty et al., 2005) including anti diabetic and anti hypertensive activity (Kwon, Vatter, & Shetty, 2006). In addition, the flavonoid-rich in plants also have the ability to inhibit ACE-I activity as shown *in vitro* and *in vivo* (Actis-Goretta, Ottaviani, Keen, & Fraga, 2003; Kwon et al., 2006). The consumption of flavonoid-rich foods may thus mimic synthetic ACE-I inhibitors and provide health benefits, but without the unwanted adverse side effects of consuming synthetic drugs such as nausea, dry mouth, and diarrhea (Shetty et al., 2005). In the present studies we have studied dill (*Anethum graveolens*), peppermint (*Mentha piperita*) and basil (*Ocimum basilicum*) as plants of choice for they are rich in phytochemicals and are commonly used in food preparation.

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Dill, peppermint and basil are traditionally grown cash crops in Europe and Central Asia for the production of fresh herbage, dry leaves or essential oils (Hay & Waterman, 1993, p. 185). The products from these plants find their applications as culinary herb or as minor adjuncts to salads (fresh herbs) and herbal teas (dry leaves/shoots) (Baratta, Dorman, Deans, Biondi, & Ruberto, 1998; Lu & Foo, 2001) and as aromatic agents in the food, pharmaceutical, perfumery and cosmetic, functional food and nutraceuticals industries (essential oils). Significant amount of antioxidants (Baratta et al., 1998; Lu & Foo, 2001) and anti-microbial activity (Basilico & Basilico, 1999; Marino, Bersani, & Comi, 2001) have been demonstrated from both the extracts and essential oils from these plants. Other benefits attributed to consuming these plants include anti-cancer and anti-microbial properties for *O. basilicum* (Manosroi, Ozguner, Aydin, & Gokalp, 2004), relieve upset stomachs and to inhibit the growth of certain bacteria for *M. piperita* (Sharma & Tyagi, 1991) and stimulant for lactation, antispasmodic and carminative for *A. graveolens* (Hosseinzadeh, Karimi, & Ameri, 2002). The making of herbal-yogurts would thus contribute to the development of milk products containing plant phytochemicals. Since yogurt also exhibits several of these herbs functional properties we have explored the potentials of adding these herbs to further enhance yogurt functional values. Previous reports have shown crucial influence of additives on the nutritional and functional values of yogurt (e.g., pH and proteolysis (Papadimitriou et al., 2007), eating quality (Barrantes, Tamime, Muir, & Sword, 1994) and ACE-1 inhibition (Ramchandran & Shah, 2010) and the antioxidant potential (Mariken et al., 2002)). Thus, the aims of the present studies were to investigate the effects of mixing dill, peppermint and basil with milk on fresh and refrigerated yogurt in relation to (1) acidification of yogurt, (2) TPC and antioxidant activities (3) hydrolysis of milk protein and (4) inhibition of ACE-1 *in vitro*.

2. Materials and methods

2.1. Materials

2.1.1. Herbs

The three herbs (*M. piperita*, *A. graveolens* and *O. basilicum*) used in the present studies were cultivated in Tabriz (Northwest of Iran) during summer. They were purchased in the same year in dried form from a local shop in Iran. These were dried to constant weight and ground to pass through 1 mm screen. Ground herbs were kept in clean and dry air tight dark glass bottles and the bottle were placed at room temperature away from direct sunlight.

2.1.2. Starter culture

Starter culture was prepared by inoculating 1 L pasteurized full cream milk (4%) with a sachet of the following yogurt bacteria mixture: *Lactobacillus acidophilus* LA-5, *Bifidobacterium bifidum* Bb-12, *Lactobacillus casei* LC-01 and *Streptococcus thermophilus* Th-4 in the ratio of 4:4:1:1 (Chris Hansen, Denmark) and a capsule of probiotic mix containing *L. bulgaricus*, *L. rhamnosus*, *B. infantis* and *B. longum* in the ratio of (1:1:1:1) (Bio-Life, Malaysia). The milk bacteria mixture was incubated at 41 °C for 12 h and the yogurt formed was stored at 4 °C and used as starter culture within 2 weeks (Rashid, Togo, Ureda, & Miyamoto, 2007). We routinely found the pH of the starter culture to range between 4.1–4.3 and viable bacteria to range between $2.0\text{--}5.0 \times 10^6$ cfu/g and $6.0\text{--}10.0 \times 10^8$ cfu/g for *Lactobacillus* spp. and *S. thermophilus* respectively which were slightly lower than those reported by Buyong, Kok, and Luchansky (1998) on day 14 of storage.

2.2. Preparation of herbal water extract

Water extract for the three herbs was prepared on the same day by soaking each herb in distilled water (1:10 ratio) for 12 h at 70 °C followed by centrifugation (2000 rpm, 10 min at 4 °C). The supernatant was harvested and stored at 4 °C in refrigerator. These extracts were used within 3 days for the preparation of herbal-yogurts.

2.3. Preparation of yogurt

Plain- and the three herbal-yogurts were prepared on the same day. Herbal water extract (100 mL) was added into pre-warmed (41 °C) pasteurized full cream (4%) milk (850 mL) followed by the addition of starter culture (50 mL) containing *S. thermophilus*, *L. acidophilus*, *L. bulgaricus* and *B. bifida*. Full fat milk powder (2 g; 4% fat) was added to correct the milk solid content to 15% g/mL (Shah, 2003). The extract-starter culture-milk mixture was mixed thoroughly and aliquoted (100 mL) into disposable plastic containers. Plain-yogurt was prepared essentially in the same manner as herbal-yogurt with the exception that distilled water (100 mL) was used instead of herbal extract. Yogurts were fermented in water bath (41 °C) until pH was reduced to 4.5 followed by refrigeration (4 °C) for 2 h (fresh yogurt or 0 day storage) up to 28 days.

2.4. Preparation of yogurt water extracts

Plain- and herbal-yogurts (10 g) were homogenized with 2.5 mL of sterile distilled water. The pH of the yogurts was determined and the yogurts subsequently acidified to pH 4.0 with HCl (0.1 M). The acidified yogurts were then heated in water bath (45 °C) for 10 min followed by centrifugation (5000g, 10 min 4 °C). NaOH (0.1 M) was added to adjust the pH of supernatant to 7.0. The neutralized supernatants were re-centrifuged (5000g, 10 min 4 °C) and the supernatant was harvested and stored in a –20 °C freezer until required for analysis.

2.5. pH and total titratable acid (TTA) determination

Yogurt was initially homogenized in water (1: 9 ratio) prior to pH determination. The pH of the homogenized yogurt was read using a digital pH meter (Mettler-Toledo 320, Shanghai) (Kailasapathy, 2006). TTA was determined by titration using 0.1 N NaOH. Yogurt sample (1 mL) was transferred into an Erlenmeyer flask containing 9 mL dH₂O. Several (3–5) drops of 0.1% phenolphthalein as pH indicator were added. The yogurt mixture was then titrated with 0.1 N NaOH under continuous stirring until the development of a consistent pink color. The amount of acid produced during fermentation was calculated as follows:

$$\text{Percentage of Lactic Acid} = \text{Dilution factor}(10) \times V \text{ NaOH} \\ \times 0.1 \text{ N} \times 0.009 \times 100\%$$

where *V* is volume of NaOH required to neutralize the acid.

2.6. Total phenolic assay

Total phenolic compounds were determined by an assay modified from Shetty et al. (2005). Briefly, water extracts of herbs (diluted to similar extent as for herbal-yogurt) or yogurts (1.0 mL) were mixed with 1.0 mL of 95% ethanol and 5 mL of distilled water. These solutions were mixed with 0.5 mL Folin–Ciocalteu reagent (diluted 1:1 with distilled water) and were left to stand for 5 min at room temperature. Na₂CO₃ (1.0 mL, 5% g/100 mL) was then added to the reaction mixture followed by 60 min incubation at room

temperature. Absorbance at 725 nm was converted to total phenolic (ug gallic acid equivalent, (GAE)/mL) using a regression of known concentrations of gallic acid (Sigma–Aldrich, Germany; 5–60 µg/mL in methanol) which was determined every time total phenolic assay was carried out.

2.7. Antioxidant activity by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical inhibition (DRI) assay

Yogurt extracts (250 µL) were added into 3 mL of 60 mmol/L DPPH (Sigma–Aldrich, Germany)/L in ethanol. The decrease in absorbance was monitored at 517 nm until a constant reading was obtained. The constant reading for the yogurt extracts and control (consisting of 250 µL of water in place of extract) was used in calculating the % inhibition of DPPH oxidation (Apostolidis, Kwan, & Shetty, 2007) as follows:

$$280\% \text{ inhibition} = \frac{[A_{517}^{\text{control}} - A_{517}^{\text{extract}}]}{[A_{517}^{\text{control}}]} \times 100$$

2.8. o-Phthalaldehyde (OPA) assay

Proteolysis products in yogurt water extract were measured using o-phthalaldehyde (OPA) which reacts with primary amines (functional group of peptides) in the presence of mercaptoethanol (Church, Swaisgood, Porter, & Catignani, 1983). Sodium tetraborate (Borax; 25 mL 100 mM), 2.5 mL 20% (wt/wt) sodium dodecyl sulfate and 1.1 mL OPA (40 mg OPA dissolved in 1 mL methanol + 100 µL β-mercaptoethanol) (Sigma–Aldrich, Germany) were mixed in 21.4 mL dH₂O, (Goodno, Swaisgood, & Catignani, 1981). This reagent, prepared fresh on the day of assay, was protected from direct light and used within 2 h of preparation. Aliquots of yogurt water extract (30 µL containing 5–100 µg proteins) were added directly to OPA reagent (1.0 mL) in a 1.5 mL quartz cuvette and the solutions were mixed briefly by inversion prior to 2 min incubation at room temperature followed by absorbance (340 nm) measurement. The peptide concentration was estimated against the tryptone (Difco Laboratories, Sparks, MD, USA) standards (0.125–1.50 mg/mL).

2.9. Angiotensin-I converting enzyme (ACE) inhibitory activity

Inhibition of ACE by yogurt was determined using the spectrophotometric (Thermo spectronic 10 UV (190–1100 nm, USA) method based on the hydrolysis of a furanocryloyl tripeptide (FA–Phe–Gly–Gly (FAPGG), Sigma Aldrich, St Louis, MO, USA) to FA–Phe (FAP) and the dipeptide Gly–Gly (Vermeirssen, Camp, & Verstraete, 2002). Yogurt water extracts (300 µL) were mixed with ACE reagent (500 µL; 1.0 mmol/L FAPGG in 50 mmol/L Tris–HCl with 400 mmol/L NaCl, pH 8.3) in a cuvette. The mixture was mixed thoroughly and incubated in a water bath (37 °C) for 2 min. Rabbit lung extract (300 µL) in 50 mmol/L Tris–HCl, pH 8.3 was added and the mixture was mixed thoroughly followed by initial absorbance reading at 340 nm. The enzymatic reaction was allowed to take place at 37 °C for a total period of 20 min with brief absorbance reading every 5 min. The absorption decrease per minute (an expression for ACE activity) was linear during the last 15 min of incubation and was used in the calculation of enzyme activity. The extent of ACE inhibition was calculated (Vermeirssen et al., 2002) as follows:

$$\text{ACE - inhibition}(\%) = [1 - ((C - D)/(A - B))] \times 100$$

where A = absorbance of the ACE solution in the buffer; B = absorbance of the buffer; C = absorbance of the ACE solution

with the ACE-inhibitory component added in the buffer; D = absorbance of the ACE-inhibitory component in the buffer.

2.10. Preparation of rabbit lung extract

Fresh rabbit lung were snap-frozen to –20 °C followed by grinding using pestle and mortar and subsequently homogenized with ice-cold 0.05 M potassium phosphate buffer, pH 8.3 (Vermeirssen et al., 2002). The homogenized rabbit lung was then centrifuged for 60 min at 20000 g. The clear red supernatant possessed high ACE activity and was aliquoted into 1.0 mL ampoules prior to freezing (–20 °C) until required for further analysis.

2.11. Statistical analysis

A total of three separate experiments were carried out and assays were performed in triplicate. Data were expressed as mean ± standard deviation and the data were analyzed using SPSS 14.0 (Chicago, IL, USA) for Windows. General Linear Model procedures and Tukey test for means comparison were used for determining significant difference at $p < 0.05$.

3. Results and discussion

3.1. Effects of herbs on yogurt fermentation

All yogurts had similar initial pH readings (6.42–6.50; Fig. 1), despite differences ($p < 0.05$) in herbal water extract pH (6.69, 7.30 and 7.10) and TTA (0.20, 0.16 and 0.22% lactic acid equivalent) values for *O. basilicum*-, *M. piperita*- and *A. graveolens*-yogurts respectively. The pH of plain-yogurt decreased in a linear manner during 90–240 min of fermentation (0.4 pH unit/h), whereas all herbal-yogurts had faster pH decrease during 90–180 min (~0.6 pH unit/h) than during the 180–240 min (~0.3 pH unit/h) of fermentation. The pH reduction rates of herbal-yogurts (–0.60 ± 0.04, –0.60 ± 0.06 and –0.66 ± 0.07 pH unit/h for *O. basilicum*-, *M. piperita*- and *A. graveolens*-yogurt respectively) were faster than that of plain-yogurt (–0.42 ± 0.05 pH unit/h; $p < 0.05$) during the 90–180 min of fermentation. This resulted in earliest pH 4.5 to be reached by *A. graveolens*-yogurt (240 min) followed by *O. basilicum*-, *M. piperita*- and plain-yogurts (300, 300 and 390 min respectively). The presence of herbs appeared to enhance the metabolic activity of yogurt bacteria. pH is a measure of H⁺ concentration contributed by the production of organic acids by

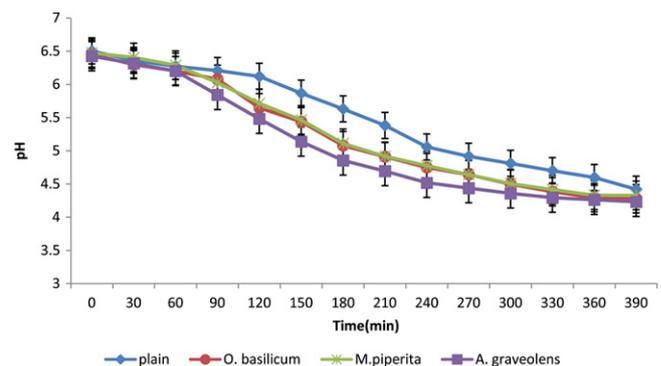


Fig. 1. pH profiles during the fermentation process of yogurts in the absence or presence of *O. basilicum*-, *M. piperita*- and *A. graveolens*-herbal water extracts at 41 °C. Plain-yogurt prepared from pasteurized homogenized milk standardized to 15 g/100 mL total solids containing 50 mL/L starter culture. Herbal-yogurt was plain-yogurt which added with 100 mL herbal water extract and 2 g full fat milk powder (4% fat) per 1 L yogurt. Each experiment was repeated three times and values are means ± SD.

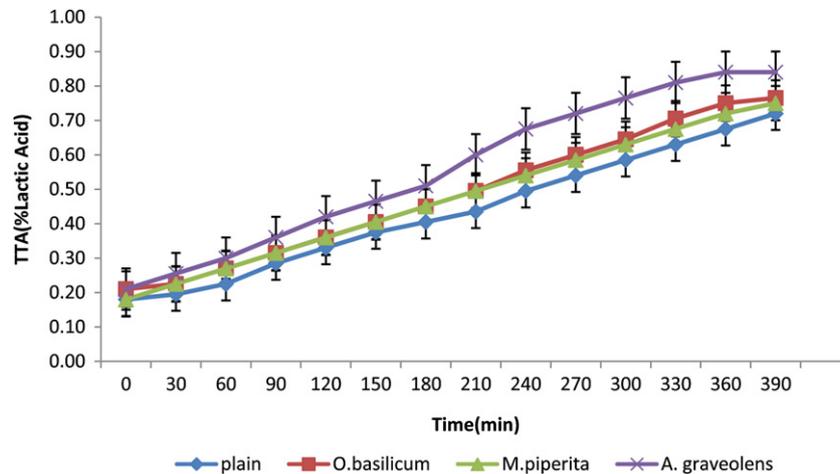


Fig. 2. Changes in total titratable acid (% lactic acid) during the fermentation process of yogurts in the absence or presence of *O. basilicum*-, *M. piperita*- and *A. graveolens*-herbal water extracts at 41 °C. Each experiment was repeated three times and values are means \pm SD.

lactic acid bacteria (LAB). The fermentation of yogurt by conventional starter culture occurred by the symbiotic activities of two groups of bacteria. *Lactobacillus* spp. proteolytic activity produces amino acids and small peptides that stimulate *S. thermophilus* growth (Sandine & Elliker, 1970) whereas *S. thermophilus* metabolites, carbon dioxide and formic acid stimulate the growth of *Lactobacillus* spp. (Kailasapathy, 2006). Extended refrigeration to 28 days decreased the pH of yogurts to lower pH values (4.2–4.4) possibly as a result of accumulation of acetic acid, acetaldehyde, formic acid and lactic acid (Vedamuthu, 1982). TTA, which measures the equivalent percentage (%) of lactic acid present in the yogurt during fermentation, is shown in Fig. 2. The increase in TTA for the plain-yogurt was almost linear in comparison to the sigmoidal curve for pH changes. The TTA for the three herbal-yogurts was higher than plain-yogurt at all time points during the incubation. *A. graveolens*-yogurt showed the highest rate of TTA production 0.163 ± 0.005 followed by *O. basilicum*-, plain- and *M. piperita*-yogurts (0.101 ± 0.003 , 0.094 ± 0.002 and $0.089 \pm 0.001\%/h$ respectively) between the 180th and 240th minute of fermentation. Total titratable acidity is the total amount of hydrogen ions present in the fermented milk sample with the exception of those bound to alkaline ions. The determination of total titratable acid (TTA) thus is more relevant in the evaluation of fermentation capacity of microbes (Geidam, Ambali, & Onyelili, 2007). Organic acids produced in yogurt (lactic acid, citric acid, formic acid, acetic acid and butyric acid (Ostlie, Treimo, & Narvhus, 2003)) were reported to be linearly related

with the accumulation of TTA (Billard, Mekki, Ouadi, & Gaillard, 2007). Variations in titratable acidity that occur in yogurts in the present study can thus be associated to differential microbial population during fermentation (Eissa, Mohamed Ahmed, Yagoub, & Babiker, 2010). Evaluation by 12 untrained panels showed the presence of herbs imparted unique organoleptic properties to yogurt. In comparison to plain-yogurt, *M. piperita*-yogurt was considered best ($p > 0.05$) for aroma and overall performance, whereas flavor score was highest ($p < 0.05$) for *A. graveolens*-yogurt and lowest ($p < 0.05$) for *O. basilicum*-yogurt (results not shown). These results are expected because each herb under study contains several specific aromatic compounds.

3.2. Total phenolic content

The total phenolic content (TPC) of herbs were 25.6 ± 3.7 , 24.5 ± 2.9 , 30.3 ± 5.1 $\mu\text{gGAE}/\text{mL}$ for *M. piperita*, *A. graveolens*, *O. basilicum* respectively. Highest (TPC) in yogurt was found in *A. graveolens*-yogurt (35.2 ± 2.4 $\mu\text{gGAE}/\text{mL}$) followed by *M. piperita*-, *O. basilicum*- and plain-yogurts (26.6 ± 3.8 , 20.1 ± 1.4 , 15.4 ± 0.8 $\mu\text{gGAE}/\text{mL}$ respectively). The TPC increased gradually for all herbal-yogurts during refrigeration with maximum values of 47.7 ± 1.6 , 37.5 ± 2.6 , 28.6 ± 2.1 and 19.5 ± 1.5 $\mu\text{gGAE}/\text{mL}$ were present in *A. graveolens*-, *M. piperita*-, *O. basilicum*- and plain-yogurts respectively (Fig. 3). Since plain-yogurt contains no plant extracts, the TPC values in plain-yogurt reflect phenolic compounds related to milk protein breakdown (Damin, Alcántara, Nunes, &

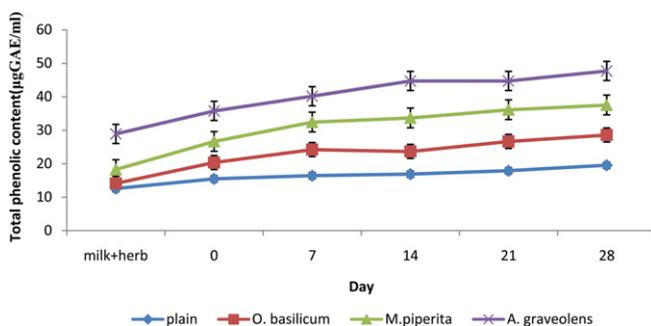


Fig. 3. Total phenolic concentration ($\mu\text{gGAE}/\text{mL}$) in plain- and herbal-yogurts during refrigerated (4 °C) storage. Each experiment was repeated three times and values are means \pm SD. Total phenols were expressed as μg gallic acid equivalent ($\mu\text{gGAE}/\text{mL}$).

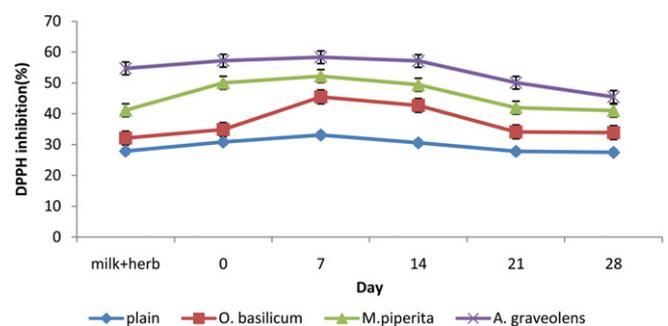


Fig. 4. Antioxidant capacity (% inhibition of DPPH oxidation) by plain- and herbal-yogurts during 28 day of refrigerated (4 °C) storage. Each experiment was repeated three times and values are means \pm SD.

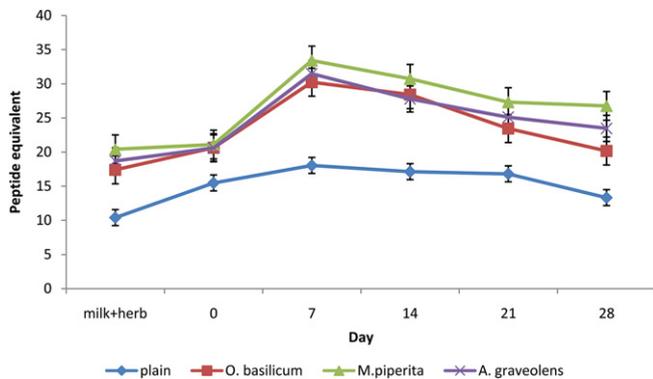


Fig. 5. Changes in o-phthalaldehyde (OPA) values in plain- and herbal-yogurts after fermentation (41 °C) and during refrigerated (4 °C) storage for 28 days. Plain-yogurt prepared from pasteurized homogenized milk standardized to 15 g/100 mL total solids containing 50 mL/L starter culture. Herbal-yogurt was plain-yogurt which added with 100 mL herbal water extract and 2 g full fat milk powder (4% fat) per 1 L yogurt. "0" day represents OPA values after fermentation at 41 °C. Each experiment was repeated three times and values are means \pm SD.

Oliveira, 2009). The amino acid tyrosin for instance has a phenolic side chain suggested (Shah, 2000) to give rise to the reading in TPC. Another possibility is that microbial utilization of phenolic acids such as ferulic and p-coumaric acid during fermentation process and post acidification lead to the production of other phenolic acids such as vanillic and p-hydroxybenzoic acids before the aromatic ring structure is broken down (Blum, 1998). The increased TPC in herbal-yogurts can be explained by the presence of indigenous phytochemical compounds in herbs during storage period (e.g., flavonoids and phenolic compounds) in *A. graveolens* (Ishikawa, Kudo, & Kitajima, 2002).

3.3. DPPH scavenging activity

All herbal-yogurts had higher ($p < 0.05$) antioxidant activity than plain-yogurt, both at the end of fermentation and throughout the storage period. Inhibition of DPPH oxidation by each yogurt increased to highest values of 58.7 ± 3.9 , 52.3 ± 1.8 , 45.2 ± 4.4 and $33.6 \pm 0.8\%$ for *A. graveolens*-, *M. piperita*-, *O. basilicum*- and plain-yogurts respectively on day 7 of refrigerated storage followed by gradual reduction between 28% and 46% by day 28 of storage (Fig. 4). The higher antioxidant activities in herbal-yogurts than in plain-yogurt were most likely contributed by individual herbal phytochemical contents and as a result of microbial metabolic activities (Thompson, Lopetcharat, & Drake, 2007). *A. graveolens* is rich in flavanoids and tannins (Shan, Cai, Sun, & Corke, 2005), whereas *M. piperita* and *O. basilicum* contain high α -tocopherol, β -carotene and ferulic acid (Ismail, Marjam, & Foong, 2004). Highest DPPH inhibition after 7 days of refrigeration may be attributed to the metabolically active yogurt bacteria even at low temperature (Papadimitriou et al., 2007). Continued microbial

growth during refrigerated storage may have altered some of the phenolic compounds and hence their antioxidant activities (Blum, 1998). The reduction in antioxidant activities during refrigerated storage of yogurt is attributed to increasing degradation of phenolic compounds with antioxidant activities (Yildiz & Eydurhan, 2009) and/or increasing milk protein–polyphenol interaction (Yuksel, Avci, & Erdem, 2010). In this regard, the consumption of yogurt is highly advisable within 7 days after yogurt-making to benefit from high live bacterial contents (Water, Keen, & Gershwin, 1999) and high antioxidant activities useful for protective cardiovascular effect (Massey, 2001).

3.4. Evaluation of proteolysis by o-phthalaldehyde (OPA) assay

All herbal-yogurts had higher OPA values (~ 20 mg/mL) than plain-yogurt (15 mg/mL) on day 0 of storage. Proteolysis in herbal-yogurts increased (28–36%) on day 7 of storage with the peptide concentration recorded for *M. piperita*-yogurt (33.4 ± 0.5 mg/mL) followed by *A. graveolens*- and *O. basilicum*-yogurts (31.5 ± 1.5 and 30.2 ± 1.9 mg/g respectively; $p > 0.05$). The OPA values in herbal-yogurts on day 28 of storage (26.8 ± 1.5 , 23.5 ± 0.4 and 20.1 ± 1.7 mg/mL for *M. piperita*-, *A. graveolens*- and *O. basilicum*-yogurts respectively) remained higher than plain-yogurt (13.3 ± 0.5 mg/mL). *L. acidophilus* and *S. thermophilus* are metabolically active even at 4 °C (Papadimitriou et al., 2007) and thus the extent of proteolysis by these bacteria could have been enhanced by the presence of herbs as shown by a substantial increase in OPA values in yogurt after the first 7 days of refrigerated storage (Fig. 5). This may lead to alteration of protein breakdown with potentially higher production of bioactive peptides (Shahidi & Zhong, 2008). The OPA value in plain-yogurt during refrigerated storage was relatively unchanged over the next 14 days but it was reduced in herbal-yogurts (Fig. 5). Although a reduction in OPA values suggests a decrease in α -amino groups reacting with β -mercaptoethanol, thus lower absorption at 340 nm, it does not necessarily implicate significant utilization of amino acids by the yogurt bacteria. Degradation of amino acids to yield carbon skeletons that are used as nutrients by LAB (Meisel, Goepfert, & Gunther, 1997) is more likely to occur during fermentation at 41 °C rather than during storage at 4 °C. The extent of proteolysis and the sizes of peptides produced from the fermentation are expected to be different in the presence of these herbs and these need to be further studied in the future.

3.5. Angiotensin-I converting enzyme (ACE)

The angiotensin-I converting enzyme (ACE) assay was carried out to evaluate the ACE-inhibitory activity of bioactive peptides produced during yogurt formation and refrigerated storage (Gobbetti, Ferranti, Smacchi, Goffredi, & Addeo, 2000). The ACE inhibition, highest by day 7 of storage but which reduced gradually thereafter to reach the lowest level on day 28 of storage (Table 1), coincided with similar changes in OPA values. This suggest that the

Table 1
Angiotensin-I converting enzyme (ACE-I) inhibition (%) by water extract (300 μ L) of plain- and herbal-yogurts during refrigerated (4 °C) storage.

Type of yogurt	0	7	14	21	28
Plain	44.3 \pm 1.9 ^{ax}	63.7 \pm 3.6 ^{ay}	53.7 \pm 4.7 ^{ay}	42.7 \pm 3.1 ^{ax}	28.8 \pm 8.8 ^{ay}
<i>M. piperita</i>	84 \pm 3.6 ^{bx}	91 \pm 2.7 ^{by}	87.3 \pm 2.3 ^{bx}	74 \pm 4.2 ^{by}	63.6 \pm 5.5 ^{by}
<i>O. basilicum</i>	60 \pm 2.6 ^{bx}	66.9 \pm 1.7 ^{bx}	62.8 \pm 4.5 ^{bx}	54.4 \pm 3.6 ^{by}	47.9 \pm 8.4 ^{by}
<i>A. graveolens</i>	66.7 \pm 9.7 ^{bx}	74.1 \pm 6.7 ^{bx}	65.6 \pm 6.3 ^{bx}	57.1 \pm 6.7 ^{by}	46.8 \pm 4.9 ^{by}

Plain-yogurt prepared from pasteurized homogenized milk standardized to 15 g/100 mL total solids containing 50 mL/L starter culture. Herbal-yogurt was plain-yogurt added with 100 mL herbal water extract and 2 g full fat milk powder (4% fat) per 1 L yogurt.

Each experiment was repeated three times and values are means \pm SD.

^{ax}Means in the same row with different alphabets are significantly different ($p < 0.05$) for each type of yogurt.

^{ay}Means in the same column with different alphabets are significantly different ($p < 0.05$) for the same storage day.

relatively less specific peptides produced during fermentation were further cleaved to smaller and more bioactive proteins during the first 7 days of refrigeration and that extensive proteolysis of these protein during extended storage up to (day 28) produced much smaller and less bioactive proteins. FitzGerald et al. (2004) also attributed an enhancement of anti-ACE with storage at 4 °C to continuous formation of many fragmented peptides as a result of exopeptidase activities on milk proteins.

In the present studies all herbal-yogurts showed higher anti-ACE activities than plain-yogurt at all storage periods ($p < 0.05$). *M. piperita*-yogurt showed the highest ACE inhibition followed by *A. graveolens*-, *O. basilicum*- and plain-yogurts. ACE enzymatic activities can be altered by the binding of biomolecules such as phytochemicals (Liu & Finley, 2005), synthetic drugs (Shen, Lukacher, Billet, Williams, & Bernstein, 2008) or bioactive peptides (Kilpi, Kahala, Steele, Pihlanto, & Joutsjoki, 2007) on the enzyme active binding sites. Since the water extract of herbs on their own (data not shown) did not affect ACE activities, these herbs may have indirectly altered the hydrolysis of milk proteins by affecting the yogurt bacterial growth and metabolism (Ayar, 2002). Proteolysis is the most important biochemical process occurring during fermentation and storage (Tamine & Deeth, 1980). Milk proteins, especially caseins are important source of bioactive peptides several of which have ACE-inhibitory peptides encrypted within their primary structures (Meisel, 1998; Saxelin, Korpela, & Mäyrä-Mäkinen, 2003) which can be released by enzymatic hydrolysis during gastrointestinal digestion or food processing (Hata, 1996; Seppo, Jauhiainen, Poussa, & Korpela, 2003). Fermented milk products, reported to contain the bioactive peptides valyl-prolyl-proline (Val-Pro-Pro) and isoleucyl-prolyl-proline (II-Pro-Pro) (Nakamura, Yamamoto, Sakais, & Takano, 1995) may act as ACE inhibitor which will bind to the enzyme competitively and prevent the breakdown of substrate furanacrylyl-Phe-Gly-Gly (FAPGG) to the products, furanacrylyl-Phe (FAP) and Gly-Gly. Future studies on the characterization of specific peptides generated during fermentation and refrigerated storage in the presence of herbs need to be carried out to support the potential uses of these herbal-yogurts with anti-ACE activities together with conventional drug treatments to assist people with hypertension.

4. Conclusion

The presence of herbal extracts altered the yogurt bacteria fermentation of milk leading to enhanced acidification of yogurts, formation of peptides and inhibition of ACE. The proteolytic activity of yogurt bacteria during fermentation and refrigerated storage was highest in the presence of *M. piperita* followed by *A. graveolens* and *O. basilicum*. The increased formations of peptides during storage coincide well with increased inhibition of ACE *in vitro*. These herbal-yogurts, by virtue of the presence of herbs and bioactive peptides, and increased antioxidant activities may offer new range of yogurts with desirable multifunctional health effects to consumers with hypertension.

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