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ORIGINAL PAPER

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THE USE OF NANO-SIZED EGGSHELL POWDER FOR CALCIUM FORTIFICATION OF COW'S AND BUFFALO'S MILK YOGURTS

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ABSTRACT

Background. Calcium is an essential element for the growth, activity, and maintenance of the human body. Eggshells are a waste product which has received growing interest as a cheap and effective source of dietary calcium. Yogurt is a food which can be fortified with functional additives, including calcium. The aim of this study was to produce yogurt with a high calcium content by fortification with nano-sized eggshell powder (nano-ESP).

Materials and methods. Nano-sized ESP was prepared from pre-boiled and dried eggshell, using a ball mill. Yogurt was prepared from cow's milk supplemented with 3% skimmed milk powder, and from buffalo's milk fortified with 0.1, 0.2 and 0.3% and 0.1, 0.3 and 0.5% nano-ESP respectively.

Results. Electron microscopic transmission showed that the powder consisted of nano-sized crystalline structures (~10 nm). Laser scattering showed that particles followed a normal distribution pattern with z-average of 590.5 nm, and had negative zeta-potential of –9.33 ±4.2 mV. Results regarding changes in yogurt composition, acid development, calcium distribution, biochemical changes, textural parameters and sensory attributes have been presented and discussed.

Conclusion. The addition of up to 0.3% nano-ESP made cow and buffalo high-calcium yogurts with an acceptable composition and quality. High-calcium yogurt may offer better health benefits, such as combating osteoporosis.

Keywords: yogurt, eggshell, nano-size powder, biochemical changes, calcium distribution, textural properties, sensory attributes

INTRODUCTION

Calcium is an essential element for the growth, activity, and maintenance of the human body. It is responsible for building the skeleton and for the effective performance of several physiological activities. A large daily intake of calcium is needed throughout the lifespan to meet nutritional requirements. However, a typical dietary calcium intake is not sufficient to satisfy the recommended daily calcium intake for all age groups. This means that individuals should take calcium from external sources in the form of calcium supplements or from calcium-fortified foods. Several natural sources of calcium carbonate, such as oyster shell, are used now for this purpose (Ahmed et al., 2015; Lee et al., 2017).

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Eggshell is a waste product that has received growing interest as a cheap and effective source of dietary calcium. The shell makes up about 11% of the total egg mass (about 6 g) and contains approximately 2.200 g of calcium, as well as other minerals such as potassium, magnesium and iron, and extracts such as collagen. Schaafsma et al. (2000) found that the eggshell powder (ESP) contains high levels (mean ±standard deviation) of Ca (401 ±7.2 mg/g) and Sr (372 ±161 µg/g), and very low levels of Pb, Al, Cd, Hg, Fe, Cu, Zn, Se and Cr. Also, it contains a small protein fraction rich in Glutamine and Argenine. A pilot study (Schaafsma and Pakan, 1999) indicated that consuming chicken ESP enriched dairy-based supplement increased the bone mass density (BMD) of subjects with a low bone mass in the short term and delayed bone demineralization for a longer period. An in vitro study (Rovenský et al., 2003) showed that ESP stimulates chondrocyte differentiation and cartilage growth, while clinical studies have shown that ESP reduces pain and osteoresorption, increases mobility and bone density, and arrests the loss of bone density in postmenopausal women and women with senile osteoporosis. The bioavailability of calcium from ESP was found to be similar or better than that of food-grade purified calcium carbonate. Several patents have been granted to nutritional and clinical applications of eggshell (Cordiero and Hincke, 2011), which shows the feasibility of using ESP as a source of dietary calcium. ESP has been used as a supplement in the production of milk tablets (Lee et al., 2016) without any significant effect on the hardness or sensory properties of the product.

Yogurt can be fortified with many functional food additives, such as calcium. The acidity developed during the fermentation of yogurt increases ionized calcium and its intestinal absorption (Unal et al., 2005). Pirkul et al. (1997) reported that Ca content of yogurt can be increased by 34–39% without marked effect on the quality of the yogurt. Both soluble and insoluble calcium salts have been used for the fortification of dairy products, but insoluble salts such as calcium carbonate and calcium phosphate have the least effect on the properties of the fortified product (Omoarukhe et al., 2010). However, the sizes of the insoluble calcium salts should be reduced in order to avoid the formation of sediment and development of a grainy texture (Fleury et al., 1998).

With the advent of nanotechnology techniques, the sizes of the insoluble calcium salts can be reduced to nano-sizes, which can increase their bioavailability. Recent studies have demonstrated that nanosizing enhances the bioavailability of insoluble calcium salts (Park et al., 2008). To the improve in their biocompatibility, nano-sized calcium can be used in bio-mineralization (Cai and Tang, 2008). Nano-sized powder was prepared from ESP and its physicochemical properties were compared with that of the micro ESP (Al Mijan et al., 2014b). The nano-sized ESP was characterized by high water absorption and less zeta-potential than the micro ESP. They (Al Mijan et al., 2014a) added different percentages (0.15–0.45%) of the prepared nano-ESP powder (0.15–0.45%) yogurt in order to fortify it with calcium. The obtained fortified yogurt was characterized by higher pH, high viscosity and less total bacterial count than the control, while the color parameters and sensory properties were only slightly affected by the added nano-ESP. They attributed the low bacterial count of the fortified yogurt to the antimicrobial activity of eggshell. They recommended limiting the percentage of added shell powder to ESP 0.30% to obtain calcium- fortified yogurt of acceptable quality and extended shelf life. Yogurt prepared from buffalo's milk is generally characterized by high calcium content and the addition of nano-ESP may cause different changes than those obtained in cow's milk yogurt.

The present study was undertaken to verify the effect of nano-ESP on the chemical and rheological properties of buffalo's and cow's milk yogurts.

MATERIALS AND METHODS

Materials

1. Fresh buffalo's and cow's milks were obtained from the dairy department, Cairo Univ. and were used without standardization of the fat content. Skimmed milk powder (3%) was added to the cow's milk only.
2. Imported skimmed milk powder was purchased from the local market
3. A mixed yogurt culture (DVS) of *Lactobacillus delbreukii* subsp. *bulgaricus* and *Streptococcus thermophilus* (1:1) was obtained from Ch. Hansen Lab. (Denmark). The starter culture was activated

in 10% sterilized reconstituted skimmed milk at 35°C, 24 hr before use.
4. All chemicals and reagents used are analytical grade.

Methods

Preparation of nano-sized eggshell powder (ESP). Eggshells were separated from boiled eggs (white) and the membranes were removed manually from the obtained shells. The eggshells without membranes were boiled for 10 min to inactivate any microbial contamination and then air dried. A coarse powder was prepared from the dried shells using a kitchen grinder. Nano-sized ESP was obtained by milling the coarse powder using a bone mill Model PQ-N2 Gear Drive--4-station (Across International, Livingston, NJ, USA), as described by Al Mijan et al. (2014b).

Particle size of nano-ESP. The z-average diameter and size distribution of nano-ESP were carried out at 25 ±0.1°C using Nano ZS/ ZEN3600 Zetasizer (Malvern Instruments Ltd., UK) with a He / Ne laser ($\lambda = 633$ nm), scattering angle 90°. Samples were diluted in 0.1M phosphate buffer pH 7.0 and filtered through 0.45 μm membrane (Mellipore, USA) to obtain a count rate in the appropriate range 100–450 nm, to avoid multiple scattering phenomena due to inter-particle interaction. Immediately, the diluted sample was transferred into a polystyrene cuvette for size determination, and then the z-average diameter (Dz) and particle disparity index (PDI) were recorded by dynamic light scattering (DLS) as described by Giroux et al. (2010).

Determination of zeta-potential of nano-ESP. The zeta-potential (ζ-potential) of nano-ESP was determined by laser Doppler electrophoresis at 25°C using a Malvern Zetasizer Nano ZS analyzer (Malvern Instruments Ltd., Malvern, UK). Each five-times diluted sample (1 ml) was transferred to a universal folded capillary cell equipped with platinum electrodes, and ζ-potential was determined by measuring the direction and velocity of the nanoparticles moving in the electric field and then calculated by the Zetasizer Software (Malvern Instruments Ltd., Malvern, UK) according to the Henry equation as given by (Dai et al., 2015).

Transmission electron microscopy of nano-ESP. Nano-ESP was examined by transmission electron microscopy (TEM, JEM-100 CX, JEOL, Japan) to evaluate its size and shape. A 50-fold diluted dispersion of nano-ESP in distilled water was deposited onto a carbon grid. After 30 s, the dispersion was stained with uranyl acetate solution for 1 min. The excess solution was drawn off using filter paper, and the sample was dried at room temperature. The size, shape and surface morphology of the nano particles were examined by TEM, using an electron beam of 100 kV.

Preparation of nano-ESP fortified yogurt. Nano--ESP was added to buffalo's milk, in the proportion of 0 (control), 0.1, 0.3 and 0.5% respectively, and, for cow's milk, nano-ESP was added in the proportions of 0 (control), 0.1, 0.2 and 0.3% respectively. The milks were gently stirred and left for 1 hr at room temperature to attain equilibrium. Milk from the different treatments was heated at 85°C/10 min and then cooled to 40°C, inoculated with 2% of the activated yogurt starter culture, transferred to 150 ml plastic cups, capped and then incubated at 40°C until complete coagulation (4 hrs). Samples of yogurt from the differed treatments were kept cold (7 ±2°C) for 3 weeks and analyzed when fresh and at weekly intervals. Three replicates were prepared and analyzed from the different treatments.

Chemical analysis of yogurt. Control yogurt and yogurt fortified with nano-ESP were analyzed for total solids, fat, total and soluble N and ash contents (AOAC, 2012). The pH values of yogurt from the different treatments were measured using a digital pH meter equipped with a combined electrode (Hanna, Germany) and the titratable acidity was determined as described by Ling (1963). The total free amino acid contents of yogurt from different treatments was determined as described by Folkertsma and Fox (1992), total volatile fatty acids as described by Kosikowski (1986) and acetaldehyde and diacetyl as described by Lee and Jago (1969; 1970). Total Ca++ was determined in a mixed yogurt sample while the soluble Ca++ was determined in the clear serum obtained from the mixed yogurt sample by centrifuging at 4000 g/10 min. Total and serum calcium were determined by complex metric titration using calcein as an indicator, as described by (Ntailianas and Whitney, 1964).

Textural properties. The textural properties of yogurt from different treatments were assessed using the textural analyzer (Multi test 1dMemesin, Food Technology Corporation, Slinfold, W. Sussex, UK) equipped with a 25 mm diameter perplex conical shaped probe. The texture profile analysis (TPA) was done on yogurt samples using the double compression test, which generated a plot of force (Newton, N) versus time (second, s). Compression was done at five different points on the sample surface. In the 1st stage the sample was compressed by 30% of its original depth at a speed of 2 cm/min during the pretest, compression and the relaxation of the sample. The following parameters were calculated from the force-time curve according to the definition given by the International Dairy Federation IDF (1991):

Hardness, N = maximum force of the 1st compression

Cohesiveness = area, A, under the 2nd compression / area, A, under the 1st compression (*A*2/*A*1)

Springiness, mm = length, L, of the 2nd compression / length, L, of the 1st compression (*L*2/*L*1)

Gumminess, N = hardness × cohesiveness

Chewiness index, mJ = gumminess × springiness

Sensory analysis. Yogurts from different treatments were evaluated for the following sensory attributes: color and appearance (out of 10 points), flavor including acidity, sweetness, saltiness, bitterness, and mouth feel (out of 60 points), body and texture (out of 30 points) and overall acceptability (sum of scores for different attributes). A taste panel of 8 panelists from the staff of the dairy department, NRC, Cairo was involved in the sensory analysis.

Statistical analysis. The VassarStats (http://www.faculty.vassar.edu/lowry/anova2corr.html) computing site was used to analyze the obtained data statistically by Anova of two independent variables according to Lowry (2009).

RESULTS

Characteristics of nano-ESP

Transmission electron microscopy. Figure 1 shows that the ESP consists of clusters of nano-sized crystals of particles (about 10 nm in size). These clusters had variable sizes, in the order of few µms. Ultrasound treatment of the suspension failed to disrupt the nano-ESP clusters. Very few individual crystals were visible on the micrograph.

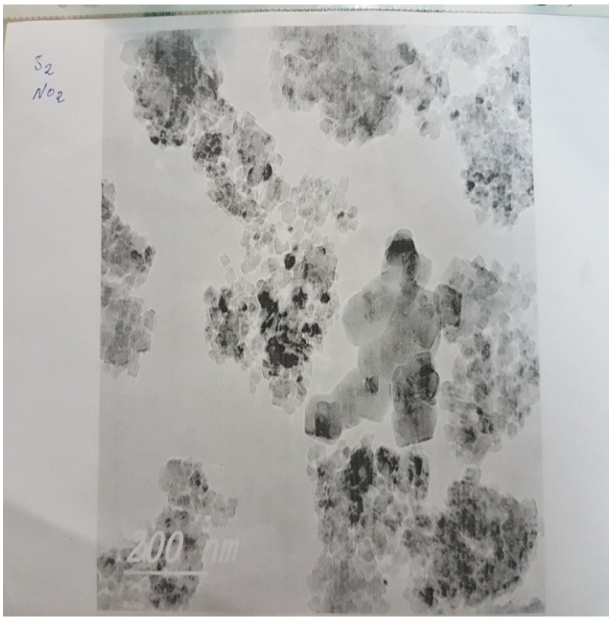

Fig. 1. TEM micrograph of ESP

Particle size distribution. Figure 2 shows the particle size distribution of nano-ESP measured by intensity. Nano-ESP behaved as a single normal distribution curve. The z-average particle size was 590.5 nm with particle disparity index (PDI) of 0.654. Even after filtration through 0.45 µm membrane filter, large clusters were apparent, indicating rapid aggregation of the nano-sized particles after filtration.

Zeta-potential. Figure 3 shows that the nano-ESP moved as a single peak in the electric field with an apparent zeta-potential of –9.33 ±4.2 mV.

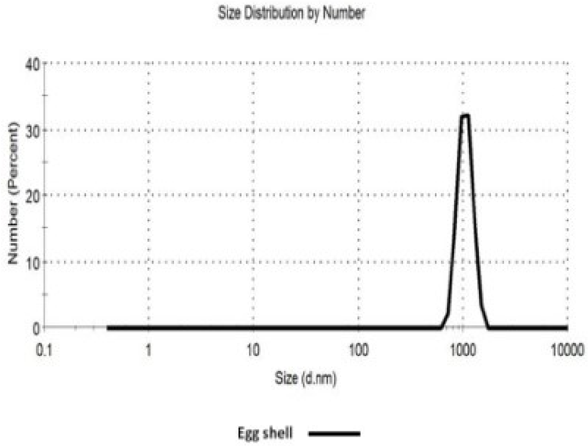

Fig. 2. Particle size distribution of nano-ESP

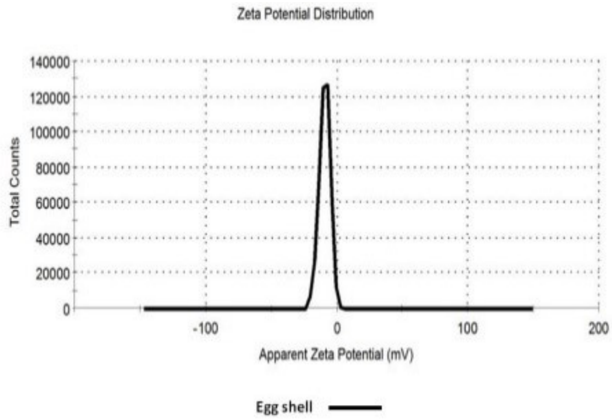

Fig. 3. Zeta-potential of nano-ESP

Effect of added nano-ESP on the manufacturing of yogurt

Preliminary studies showed that the addition of 0.3% nano-ESP to cow's milk and 0.5% to buffalo's milk gave yogurt with a firm coagulum within 4–5 hr. Addition of 0.5% nano-ESP to cow's milk retarded the fermentation time and resulted in a weak curd. Therefore, addition of nano-ESP to cow's yogurt was limited to 0.3%.

Changes in the gross composition and pH of yogurt

Table 1 shows the changes in the total solids, fat, total and soluble N, ash content, acidity and pH of cow's and buffalo's yogurts respectively, as affected by the added percentage of nano-ESP and storage period.

In both yogurts, the total solid (TS) contents increased significantly ($P < 0.05$) with the increased percentage of added nano-ESP, while the storage period had no significant effect on TS. Similar results were reported for the TS changes in commercial yogurt during storage (Assem et al., 2013). Cow's milk yogurt had higher TS than the buffalo's milk yogurt due to the addition of skimmed milk powder in the yogurt manufacturing process.

The fat content of buffalo's and cow's milk yogurts meets the (ESO, 2007) standard specification for fat content (5.5% for buffalo's and 3.0% for cow's yogurt). The fat content of buffalo's milk yogurt was not affected by the added ESP and storage period, while the fat content of cow's milk yogurt increased slightly but significantly during storage, mainly due to whey syneresis.

The total and soluble N of yogurt from cow's and buffalo's milks were not affected ($P > 0.05$) by the addition of nano-ESP or storage period.

The acidity of yogurt increased gradually, while the pH decreased in both cow's and buffalo's milk yogurts. The development of acidity was slightly faster in cow's milk yogurt than in buffalo's during fermentation and storage. In both yogurts, the addition of nano-ESP higher than 0.1% decreased acid development significantly ($P < 0.05$).

Changes in the colloidal and soluble calcium content

The changes in the total colloidal and soluble calcium content of cow's and buffalo's milk yogurts, supplemented with variable percentages of nano-ESP during storage, are presented in Table 2. The total, soluble and colloidal Ca of fresh cow's and buffalo's milk yogurts increased significantly ($P < 0.05$) as the percentage of added nano-ESP increased. As expected, buffalo's milk yogurt contained higher percentages of calcium in every form than that made from cow's milk, due to the higher Ca content of buffalo's milk. During storage, the soluble Ca increased ($P < 0.05$) while the colloidal Ca decreased ($P < 0.05$) in both cow's and buffalo's milk yogurts from the different treatments.

Table 1. Changes in the composition of cow's (C) and buffalo's (B) milk yogurts as affected by cold storage and percentage of added eggshell powder (ESP)

Storage, days	Control		0.1% ESP		0.3% ESP		0.5% ESP
	C	B	C	B	C	B	B
				Total solids, %			
Fresh	16.11^{c}	14.91^{c}	16.32^{bc}	15.30^{bc}	16.61^{a}	15.38^{ab}	15.42^{a}
5	16.43^{c}	15.02^{c}	16.65^{bc}	15.46^{bc}	16.90^{a}	15.47^{ab}	15.50^{a}
10	16.7^{c}	14.77^{c}	16.85^{bc}	14.96^{bc}	17.03^{a}	15.10^{ab}	15.55^{a}
15	16.82^{c}	14.84^{c}	16.90^{bc}	15.18^{bc}	17.13^{a}	15.30^{ab}	15.62^{a}
				Fat, %*			
Fresh	3.5^{bB}	5.8	3.6^{abB}	5.8	3.7^{aB}	5.9	5.8
5	3.5^{bB}	5.8	3.7^{aB}	5.9	3.8^{aB}	6.0	5.7
10	3.6^{bAB}	5.7	3.7^{bB}	5.7	3.9^{aAB}	5.7	5.8
15	3.7^{bA}	5.7	3.9^{aA}	5.7	4.0^{aA}	5.7	5.9
				Total, N%*			
Fresh	0.69	0.71	0.69	0.70	0.68	0.72	0.73
5	0.69	0.71	0.68	0.72	0.66	0.75	0.77
10	0.68	0.73	0.66	0.74	0.65	0.76	0.81
15	0.66	0.73	0.65	0.76	0.64	0.78	0.82
				Soluble, N%*			
Fresh	0.12	0.23	0.11	0.22	0.11	0.22	0.22
5	0.12	0.23	0.12	0.23	0.11	0.23	0.24
10	0.13	0.24	0.12	0.24	0.12	0.24	0.24
15	0.18	0.24	0.13	0.26	0.12	0.25	0.27
				Acidity, %			
Fresh	1.10^{a}	0.97^{a}	1.15^{a}	1.03^{a}	1.29^{a}	0.93^{ab}	0.88^{b}
5	1.22^{a}	1.03^{a}	1.26^{a}	1.10^{a}	1.34^{a}	1.00^{ab}	0.90^{b}
10	1.30^{a}	0.81^{a}	1.35^{a}	0.85^{a}	1.41^{b}	0.77^{a}	0.75^{a}
15	1.37^{a}	0.85^{a}	1.38^{a}	0.87^{a}	1.46^{b}	0.82^{a}	0.80^{a}
				pH			
Fresh	4.67^{A}	4.40^{A}	4.67^{A}	4.50^{A}	4.61^{A}	4.63^{A}	4.90^{A}
5	4.41^{A}	4.37^{A}	4.43^{A}	4.45^{B}	4.46^{A}	4.60^{A}	4.80^{B}
10	4.33^{A}	4.58^{A}	4.436^{A}	4.73^{A}	4.35^{A}	4.67^{A}	5.05^{A}
15	4.24^{A}	4.48^{A}	4.26^{B}	4.55^{A}	4.30^{B}	4.73^{A}	4.90^{A}
				Ash			
Fresh	0.90	0.74	0.98	0.83	1.17	0.94	1.10

Means with small letter superscripts indicate insignificant difference between rows (effect of added ESP), and means with capital letter superscripts indicate insignificant difference between columns (effect of storage) of cow's and buffalo's milk yogurts separately.
*No significant difference for the two factors.

Table 2. Changes in the total, soluble and colloidal calcium contents of cow (C) and buffalo (B) milk yogurts as affected by cold storage and percentage of added eggshell powder (ESP)

Storage, days	Control		0.1% ESP		0.3% ESP		0.5% ESP
	C	B	C	B	C	B	B
			Total calcium, mg/100 g				
Fresh	130^{dA}	158.3^{dAB}	140^{cA}	167.7^{cB}	160^{aA}	184.3^{bAB}	211.3^{aA}
5	127^{dAB}	156.0^{dB}	137^{cAB}	165.0^{cBC}	157^{aAB}	181.7^{bB}	209.0^{aAB}
10	124^{dB}	162.3^{dA}	134^{cB}	173.0^{cA}	155^{aB}	188.0^{bA}	206.3^{aB}
15	121.3^{dB}	160.3^{dAB}	132^{cB}	170.3^{cAB}	152^{aB}	186.3^{bA}	203.7^{aB}
			Soluble calcium, mg/100 g				
Fresh	50.7^{dC}	54.0^{dAB}	61.7^{cC}	56.3^{cA}	66.7^{aD}	68.0^{bB}	70.0^{aD}
5	53.3^{dB}	55.0^{dA}	64.3^{cB}	58.3^{cA}	70.0^{aC}	70.0^{bA}	73.0^{aC}
10	55.3^{dAB}	50.3^{dB}	67.0^{cA}	53.0^{cB}	73.0^{aB}	60.0^{bD}	77.0^{aB}
15	57.3^{dA}	52.0^{dB}	69.3^{cA}	54.0^{cB}	75.7^{aA}	64.0^{bC}	80.7^{aA}
			Colloidal calcium, mg/100 g				
Fresh	79.3^{cA}	104.3^{dB}	78.3^{cA}	111.3^{cB}	93.3^{aA}	117.0^{bC}	140.3^{aA}
5	73.7^{bA}	101.3^{dB}	73.0^{bA}	110.0^{cB}	87.0^{aA}	112.0^{bD}	136.0^{aA}
10	68.7^{bB}	112.0^{dA}	67.0^{bB}	119.3^{cA}	81.7^{aB}	128.0^{bA}	122.7^{aB}
15	64.0^{bB}	108.3^{dA}	62.3^{bB}	116.3^{cA}	76.7^{aB}	122.3^{bB}	123.0^{aB}

See foot note Table 1.

Biochemical changes

Table 3 shows the changes in acetaldehyde, diacetyl and total free amino acid contents and total free volatile fatty acids in cow's and buffalo's milk yogurts, as affected by the added nano-ESP and storage period. Acetaldehyde, an important constituent of yogurt flavor, increased significantly ($P < 0.05$) with increasing storage time. The addition of ESP slightly but significantly ($P < 0.05$) increased the development of acetaldehyde. Similarly, diacetyl content of buffalo and cow's milk yogurt increased during storage. The total free amino acid of yogurt increased slightly but significantly ($P < 0.05$) during storage, indicating slight proteolysis of yogurt proteins. The formation of volatile fatty acids (TVFA) in yogurt is a result of starter culture activity. During storage, the concentration of the developed TVFA in yogurt increased, remaining slightly higher in buffalo's than cow's milk yogurt. The TVFA of yogurt was significantly ($P < 0.05$) increased by both increasing storage period and increasing concentration of added nano-ESP.

Changes in the textural parameters

The changes in the textural parameters of cow's and buffalo's milk yogurts supplemented with different levels of nano-ESP and cold stored for different periods are presented in Table 4. The addition of 0.1% nano-ESP increased the hardness of fresh buffalo's milk yogurt, which was then decreased with further addition of higher concentrations of nano-ESP. The addition of 0.3% nano-ESP decreased the hardness of cow's milk yogurt while it increased the hardness of buffalo's milk yogurt. However, these changes were not significant. Storage significantly ($P < 0.05$) affected the hardness of yogurt. The hardness of buffalo's and cow's milk yogurts increased after 5 and 10 days

Table 3. Changes on some biochemical indexes of cow (C) and buffalo (B) milk yogurts as affected by added eggshell powder (ESP) and cold storage

Storage, days	Control		0.1% ESP		0.3% ESP		0.5% ESP
	C	B	C	B	C	B	B
Acetaldehyde content, μM/100 g							
Fresh	13.4^{aD}	25.0^{aB}	13.5^{aD}	25.1^{aB}	13.7^{aD}	25.9^{aB}	14.2^{aD}
5	20.8^{bC}	27.5^{bA}	21.5^{abC}	27.8^{abA}	22.1^{aC}	28.5^{aA}	23.2^{aC}
10	25.7^{aB}	13.5^{aD}	26.2^{aB}	13.7^{aD}	27.9^{aB}	14.0^{aD}	25.5^{aB}
15	31.3^{bA}	21.9^{bC}	32.4^{abA}	22.7^{abC}	33.2^{aA}	23.4^{aC}	28.3^{aA}
Diacetyl content, μM/100 g							
Fresh	7.7^{D}	18.3^{B}	7.7^{D}	18.7^{B}	8.1^{D}	19.3^{B}	8.7^{D}
5	11.7^{C}	22.2^{A}	12.2^{C}	22.1^{A}	12.8^{C}	23.4^{A}	15.0^{C}
10	18.7^{B}	8.3^{D}	17.4^{B}	8.4^{D}	20.6^{B}	8.8^{D}	19.4^{B}
15	23.2^{A}	14.0^{C}	23.5^{A}	14.5^{C}	24.6^{A}	14.9^{C}	23.3^{A}
Total free amino acid content, μg/100 g							
Fresh	15.0^{bB}	15.0^{dB}	15.5^{bB}	16.0^{cA}	16.7^{aD}	17.0^{bB}	16.5^{aD}
5	15.3^{bB}	16.0^{cA}	16.0^{bB}	16.5^{cA}	18.0^{aC}	18.0^{bA}	17.5^{aC}
10	16.3^{dB}	14.5^{bB}	17.3^{cA}	14.5^{bB}	19.7^{aB}	16.0^{aC}	18.5^{aB}
15	17.6^{cA}	14.5^{bB}	18.3^{cA}	15.0^{bB}	21.3^{aA}	17.0^{aB}	19.5^{aA}
Total volatile fatty acids, ml 0.1 N NaOH/100 g							
Fresh	13.2^{bD}	23.0^{cB}	13.7^{abD}	25.1^{bB}	14.5^{aD}	26.0^{aB}	18.9^{aD}
5	15.6^{cC}	25.8^{cA}	17.9^{bC}	27.3^{bA}	20.7^{aC}	28.1^{aA}	25.0^{aC}
10	17.7^{cB}	17.4^{bD}	20.1^{bB}	17.9^{abD}	21.9^{aB}	18.4^{aD}	26.4^{aB}
15	20.9^{cA}	21.2^{cC}	22.5^{bA}	23.1^{bC}	24.0^{aA}	24.5^{aC}	28.2^{aA}

See foot note Table 1.

of cold storage respectively, and then decreased thereafter in cow's milk yogurt, while continuing to increase in buffalo's milk yogurt.

Sensory properties

Table 5 shows the scores for the flavor, appearance, body and texture of cow's and buffalo's milk yogurts made with different levels of added nano-ESP during 15 days of cold storage. Both storage time and percentage of ESP had significant ($P < 0.05$) effects on the flavor scores of yogurt. The overall acceptability of buffalo's and cow's milk yogurts was affected by both nano-ESP level and by duration of storage. The lowest total scores were given to yogurts with high levels of nano-ESP (0.5%) and stored for 15 days.

DISCUSSION

The grinding of pre-boiled and dried eggshells using a ball mill resulted in powder with nano-sized crystals of about 10 nm in size, as apparent from the TEM examination. However, these crystals were present in

Table 4. Changes in the textural parameters of cow (C) and buffalo (B) milk yogurts as affected by cold storage and percentage of added eggshell powder (ESP)

Storage, days	Control		0.1% ESP		0.3% ESP		0.5% ESP
	C	B	C	B	C	B	B
Hardness, N							
Fresh	1.40^{A}	1.07^{A}	1.37^{A}	1.27^{B}	1.00^{AB}	1.24^{AB}	1.23^{B}
5	1.40^{A}	1.00^{A}	1.40^{A}	1.17^{B}	1.10^{A}	1.17^{B}	1.75^{A}
10	1.50^{A}	1.13^{A}	1.40^{A}	1.57^{A}	1.10^{A}	1.27^{AB}	1.40^{B}
15	1.43^{A}	1.27^{A}	1.30^{A}	1.77^{A}	0.90^{B}	1.48^{A}	1.00^{B}
Cohesiveness, B/A							
Fresh	0.54^{AB}	0.60^{A}	0.54^{AB}	0.58^{A}	0.42^{AB}	0.60^{A}	0.49^{B}
5	0.51^{B}	0.54^{A}	0.50^{B}	0.44^{C}	0.45^{AB}	0.44^{C}	0.54^{AB}
10	0.51^{B}	0.54^{A}	0.58^{A}	0.51^{B}	0.47^{A}	0.47^{BC}	0.57^{A}
15	0.60^{A}	0.59^{A}	0.50^{B}	0.58^{A}	0.39^{B}	0.51^{B}	0.44^{B}
Springiness, mm*							
Fresh	0.59^{A}	0.76	0.67^{AB}	0.72	0.57^{A}	0.73	0.68
5	0.51^{B}	0.62	0.62^{B}	0.64	0.53^{AB}	0.61	0.67
10	0.59^{A}	0.67	0.72^{A}	0.66	0.56^{AB}	0.54	0.55
15	0.64^{A}	0.69	0.52^{C}	0.70	0.51^{B}	0.64	0.49
Gumminess, N							
Fresh	0.77^{A}	0.67^{AB}	0.73^{AB}	0.73^{BC}	0.34^{A}	0.80^{A}	0.61^{B}
5	0.73^{A}	0.52^{B}	0.70^{AB}	0.52^{C}	0.43^{A}	0.52^{B}	0.95^{A}
10	0.77^{A}	0.60^{AB}	0.78^{A}	0.79^{B}	0.45^{A}	0.59^{B}	0.86^{A}
15	0.85^{A}	0.75^{A}	0.65^{B}	1.02^{A}	0.43^{A}	0.76^{A}	0.32^{C}
Chewiness, m/N*							
Fresh	0.46^{AB}	0.49	0.49^{A}	0.52	0.19^{A}	0.60	0.42
5	0.37^{B}	0.38	0.38^{A}	0.50	0.21^{A}	0.34	0.64
10	0.45^{AB}	0.39	0.50^{A}	0.54	0.25^{A}	0.32	0.61
15	0.56^{A}	0.52	0.41^{A}	0.72	0.19^{A}	0.50	0.32

See foot note Table 1.

clusters of larger sizes in the order of few μms. This was also apparent from the large average size of nano--ESP as measured by laser scattering. Even after filtration through 0.45 μm membrane filter, large clusters were apparent, indicating rapid aggregation of the nano-sized particles after filtration. Also, ultrasound treatment of the aqueous nano-ESP suspension failed to disrupt these clusters, which can be attributed to the hydrophobic nature of these crystalline particles. The low zeta-potential of ESP confirmed this hypothesis.

Table 5. Changes in scores for the sensory attributes of cow (C) and buffalo (B) milk yogurts as affected by cold storage and percentage of added eggshell powder (ESP)

Storage, days	Control		0.1% ESP		0.3% ESP		0.5% ESP
	C	B	C	B	C	B	B
Flavor, 60							
Fresh	57.0^{aA}	57.0^{aA}	56.7^{aAB}	59.0^{aA}	55.7^{aA}	58.0^{aA}	59.0^{aA}
5	55.0^{bB}	55.7^{bB}	57.3^{aA}	57.7^{aA}	55.3^{bA}	56.0^{aB}	57.0^{bA}
10	55.0^{aB}	58.3^{aA}	56.0^{aAB}	59.8^{aA}	56.7^{bA}	59.8^{aA}	54.3^{bB}
15	53.0^{bC}	57.7^{bA}	55.3^{aB}	59.6^{aA}	51.7^{cB}	59.3^{aA}	52.3^{cB}
Body and texture, 30							
Fresh	30.0^{aA}	30^{A}	29.7^{aA}	30^{A}	26.3^{aA}	27.7^{A}	29.0^{A}
5	30.0^{aA}	29^{A}	30.0^{aA}	29.3^{A}	25.7^{bA}	26.3^{B}	28.3^{A}
10	28.7^{abA}	30^{A}	30.0^{aA}	30^{A}	25.3^{cA}	28.7^{A}	27.3^{A}
15	27.0^{abB}	30^{A}	28.3^{aB}	30^{A}	24.7^{cB}	28.0^{A}	26.6^{B}
Color and appearance, 10							
Fresh	10.0^{a}	10^{a}	10.0^{a}	10^{a}	10.0^{a}	10^{a}	10^{a}
5	10.0^{a}	9.3^{b}	10.0^{a}	10^{a}	10.0^{a}	9.3^{b}	10^{a}
10	10.0^{a}	10^{a}	10.0^{a}	10^{a}	9.7^{a}	10^{a}	10^{a}
15	9.7^{b}	10^{a}	10.0^{a}	10^{a}	8.7^{b}	10^{a}	9.3^{b}
Total scores, 100							
Fresh	95.7^{aA}	97.0^{aA}	96.3^{aA}	99.7^{aA}	92.0^{bA}	95.7^{aA}	98.0^{aA}
5	95.0^{aA}	94.0^{aA}	97.3^{aA}	97.0^{aA}	91.0^{bA}	92.0^{bB}	95.3^{bA}
10	92.7^{bA}	98.3^{aA}	96.0^{aA}	99.8^{aA}	91.7^{bA}	98.5^{aA}	94.0^{bA}
15	89.7^{bA}	97.7^{aA}	93.7^{aA}	99.7^{aA}	88.3^{bB}	97.3^{aA}	87.7^{cB}

See foot note Table 1.

This can be explained by the difficulty dispersing the nano-ESP in aqueous solution, as seen from the TEM examination. A previous study (Al Mijan et al., 2014a) reported less particle nano-ESP (100–200 nm) which may be attributed to differences in the method used for the measurement of particle sizes. Preliminary studies showed that the addition of nano-ESP up to 0.5% had no effect on the coagulation and sensory properties of buffalo's milk yogurt, but the addition of 0.5% nano-ESP to cow's milk yogurt delayed curd formation and developed an unacceptably weak body. Therefore, the addition of nano-ESP was limited to 0.1 and 0.3% in cow's milk yogurts. The retarded coagulation time indicates that ESP affected the growth and activity of the starter culture used. Similar results were obtained by Al Mijan et al. (2014a), which they attributed to the antimicrobial activity of nano-ESP. This was also confirmed by determination of acidity during storage. Retarded acid development was found in the yogurt by analyzing data on changes in acidity and pH. Two factors may be responsible for these findings, namely: the buffer capacity of calcium salts and the antimicrobial effect of nano-ESP. Pirkul et al. (1997) found that the addition of different concentrations of Ca salts to yogurt resulted in significantly higher pH values than the control yogurt, which can be attributed to the buffer capacity of calcium salts. The slow acid development in yogurt supplemented with eggshell was accompanied by the decrease in the count of lactic acid bacteria (Al Mijan et al., 2014a). They attributed these findings to the antimicrobial effect of nano-ESP. Also, the results show faster acid development in yogurt made from cow's milk than that made from buffalo's milk. As expected, the total calcium content of the yogurt was increased with the increased addition of nano-ESP. Also, the serum calcium increased gradually with increasing storage duration, at the expense of the colloidal Ca. During fermentation the developed acidity partially solubilizes the colloidal Ca that results in the increase of the soluble Ca. Further development of acidity during storage brings more of the colloidal Ca into solution. Previous studies (Dalgleish and Law, 1998; De la Fuente et al., 2003) showed that most of the micellar Ca goes into serum with the decrease of milk pH from 6.7 to 4.0, explaining the present findings.

Several biochemical changes occurred in cow's and buffalo's milk yogurts, including the formation of acetaldehyde and diacetyl; the main flavor compounds of yogurt. Diacetyl was present in lower levels than acetaldehyde throughout the storage, in agreement with a previous study (Routray and Mishra, 2011). Yogurt from cow's milk contained slightly higher acetaldehyde and diacetyl than buffalo's milk yogurt, and less activity in the starter culture. Also, the addition of high concentrations of nano-ESP slightly enhanced proteolysis in yogurt. Various factors have been reported to affect proteolysis in yogurt (Slocum et al., 1988) including the type of starter culture and milk used, and the total solids of yogurt. The present results indicate that the addition of nano-ESP had no adverse effect on the biochemical changes in yogurt, in agreement with the finding of (Al Mijan et al., 2014a).

The increase in the hardness of yogurt can be attributed to increased acidity, whereas the decreased hardness with increasing storage for cow's milk yogurt can be attributed to proteolysis in yogurt proteins (Domagala et al., 2005; Paseephol et al., 2008). More total free amino acids developed in cow's milk yogurt than in buffalo's milk yogurt, which supports this conclusion. Changes in the cohesiveness and gumminess of yogurts as affected by the addition of nano-ESP and storage period followed a similar pattern to hardness, while springiness and chewiness were not affected by these two factors. These results clearly indicate that the addition of nano-ESP had little effect on the textural properties of buffalo's and cow's milk yogurts. Al Mijan et al. (2014a) reported that the addition of nano-ESP increased the viscosity of yogurt in agreement with the present results.

No chalkiness was detected in yogurts with different levels of ESP, except for cow's milk yogurt containing 0.3% nano-ESP, in which slight chalkiness was detected. This can be attributed to the nano-size of the ESP used. The high fat content of buffalo's milk yogurt might have masked any changes in taste brought about by the addition of nano-ESP. Similar results were reported by (Al Mijan et al., 2014a) on the flavor attributes of yogurt with different levels of nano-ESP. Changes in the color and appearance with added ESP (Al Mijan et al., 2014a) were not found in cow's milk yogurt in the present study. The body and texture of yogurts were affected by the storage period particularly in treatments containing high levels of nano-ESP ($P < 0.05$). During storage, syneresis results in significant changes to the texture of yogurt, which may explain the present results.

CONCLUSIONS

Yogurt of high calcium content and acceptable quality and composition can be made from buffalo's and cow's milks fortified with nano-ESP. The addition of nano-ESP up to 0.3% gave cow and buffalo's milk yogurts with acceptable composition, textural properties and sensory attributes. This additive would increase the Ca content of yogurt by about 15%. The high-calcium yogurt offers better health benefits in combating osteoporosis.

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