

# Salt Reduction in a Model High-Salt Akawi Cheese: Effects on Bacterial Activity, pH, Moisture, Potential Bioactive Peptides, Amino Acids, and Growth of Human Colon Cells

Akanksha Gandhi and Nagendra P. Shah

**Abstract:** This study evaluated the effects of sodium chloride reduction and its substitution with potassium chloride on Akawi cheese during storage for 30 d at 4 °C. Survival of probiotic bacteria (*Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium longum*) and starter bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*), angiotensin-converting enzyme-inhibitory and antioxidant activities, and concentrations of standard amino acids as affected by storage in different brine solutions (10% NaCl, 7.5% NaCl, 7.5% NaCl+KCl [1:1], 5% NaCl, and 5% NaCl+KCl [1:1]) were investigated. Furthermore, viability of human colon cells and human colon cancer cells as affected by the extract showing improved peptide profiles, highest release of amino acids and antioxidant activity (that is, from cheese brined in 7.5% NaCl+KCl) was evaluated. Significant increase was observed in survival of probiotic bacteria in cheeses with low salt after 30 d. Calcium content decreased slightly during storage in all cheeses brined in various solutions. Further, no significant changes were observed in ACE-inhibitory activity and antioxidant activity of cheeses during storage. Interestingly, concentrations of 4 essential amino acids (phenylalanine, tryptophan, valine, and leucine) increased significantly during storage in brine solutions containing 7.5% total salt. Low concentration of cheese extract (100 µg/mL) significantly improved the growth of normal human colon cells, and reduced the growth of human colon cancer cells. Overall, the study revealed that cheese extracts from reduced-NaCl brine improved the growth of human colon cells, and the release of essential amino acids, but did not affect the activities of potential bioactive peptides.

**Keywords:** Akawi cheese, bioactive peptides, Caco-2 cell line, human colon cell line, salt reduction

## Introduction

Sodium chloride (common salt, NaCl) is an important food additive and it contributes to the taste, texture, and enhanced shelf life of the food product (Albarracín and others 2011). Increased intake of NaCl is associated with increased risk of hypertension, osteoporosis, and kidney stones, thus there has been a need to reduce the NaCl intake (Buemi and others 2002; Kotchen 2005; Massey 2005; Heaney 2006). Dairy products, particularly brined cheeses, contribute to increased sodium intake. However, as NaCl significantly impacts the textural and sensory properties of cheese, it is difficult to completely remove it from the product. Recent studies have shown that salt concentration (ratio of sodium chloride and potassium chloride) affects the bacterial cell membrane, viability (Gandhi and others 2014; Gandhi and Shah 2015) and proteinase activity (Ayyash and others 2013a) of common dairy bacteria. It is therefore critical to evaluate the effects of varying salt concentrations (sodium and potassium chloride) in brine solution, which impacts the activity of bacteria in cheese.

The effects of reduction or substitution of NaCl in certain cheeses have been extensively studied (Katsiari and others 1997; Mistry and Kasperon 1998; Ayyash and others 2013b). Although the study by Katsiari and others (1997) showed no significant changes in composition, texture, and sensory properties, a study

by Ayyash and Shah (2011) has revealed changes in small peptides in cheeses made using partial substitution of NaCl with KCl. In general, the type of cheese, osmotic tolerance of bacteria and salting period, are among the factors that determine the properties of the product (Gobbetti and others 1999; Flourey and others 2009). Furthermore, with the increasing use of probiotic bacteria in cheeses (da Cruz and others 2009), it is important to evaluate the effects of salt stress on survival of probiotic organisms in food matrix.

Middle Eastern brined cheeses are mostly rennet-coagulated and are categorized based on addition of and type of starter culture. In general, most of the Middle Eastern cheeses are chalky white in color, possess very mild odors and, have a strong salty and acidic taste (Tamime 2008). Among the various varieties of Middle Eastern cheeses, Braided cheese is a hard cheese, Halloumi cheese is semi-hard to hard type and, Akawi and Nabulsi have been classified as semi-hard cheeses. A common characteristic of most Middle Eastern cheeses is a high salt content (Abd El-Salam and Alichanidis 2004). Akawi cheese, which is commonly consumed in Lebanon, Jordan, Palestine, and Syria, is conventionally brined for 1 mo in 10% brine solution. There is limited information on the effects of reduction of NaCl and substitution with KCl in Middle Eastern Cheeses (Ayyash and others 2012), and this is the 1st study evaluating the effects of salt reduction on release of potential bioactive peptides and amino acids using Akawi cheese as a model for high-salt cheeses.

Fermented milk products like yogurt and cheese are well known for their bioactive properties. The release of these peptides is affected by proteolysis during ripening or brining of cheese. The

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Role of salt on bioactivities of cheese . . .

brine concentration may influence the bacterial activity and proteolysis, and in turn, the properties of the peptides released. Cytochemical studies have shown that milk-derived peptides can affect the viability and growth of cancer cells (Hartmann and Meisel 2004). It is therefore important to evaluate the effect of these peptides on the growth of colon cells. In this study since the cheese (prepared with 3 potential probiotic bacteria and 2 starter bacteria) was brined for 1 mo, we wanted to investigate whether these milk-derived peptides released during brining might impact the colon cell growth, and thus a comparative study on growth of normal and cancerous colon cells was performed. To the best of our knowledge, no study has evaluated the comparative effect of cheese extracts on the normal and cancerous human colon cells. The objectives of this study were to evaluate the effects of NaCl reduction with and without potassium chloride substitution on survival of probiotics, moisture content, pH, and formation of potential bioactive peptides (angiotensin-I converting enzyme (ACE)-inhibitory and anti-oxidant) and release of amino acids in Akawi cheese. The effect of cheese extracts on the growth of normal human colon cell line (CCD 841) and human colon cancer cell line (Caco-2) was also evaluated for selected cheese extracts.

## Materials and Methods

### Bacteria cultivation

*Streptococcus thermophilus* MS (ST), *Lactobacillus delbrueckii* ssp. *bulgaricus* ASCC 859 (LB), *Lactobacillus acidophilus* CSCC 2400 (LA), *Lactobacillus casei* ASCC 290 (LC), and *Bifidobacterium longum* CSCC 5089 (BL) were obtained from Australian Starter Culture Collection (Dairy Innovation Australia Ltd., Werribee, Vic., Australia). Bacterial cultures were stored in sterile de Mann Rogosa and Sharpe (MRS) broth (Becton Dickinson and Co., Franklin Lakes, N.J., U.S.A.) supplemented with 20% glycerol at  $-80^{\circ}\text{C}$ . Each bacterium was activated as a pure culture by 1% inoculation in sterile MRS broth followed by incubation at  $37^{\circ}\text{C}$  for 24 h. The activated organisms were used after 2 successive transfers in sterile MRS broth and 2 successive transfers in sterilized reconstituted skim milk (RSM; 12% wt/vol) (Ong and Shah 2009).

### Cheese manufacturing

Homogenized, pasteurized, full fat (3.5%) milk was purchased from Kowloon Dairy, Hong Kong. The milk was tempered at  $40^{\circ}\text{C}$  and ST, LB, LA, LC, and BL were added at 1.5% (vol/vol) each and mixed thoroughly for 2 min. Milk was coagulated after 45 min by addition of diluted (1:15) chymosin (15 mL per 5 L milk; Kosher Halal ChyMax Plus; Chr. Hansen Pty Ltd., Bayswater, Vic., Australia). After 40 min, the curd was cut into cubes ( $1\text{ cm}^3$ ) using cheese knife and was allowed to settle for 15 min. The whey was drained and curd cubes were transferred to cheesecloth for further removal of whey for 20 min. Curd pieces were wrapped in cheesecloth in small portions ( $\sim 200\text{ g}$ ) and pressed (20 kg weight per 400 g curd) for 90 min at ambient temperature using a cheese press (Mad Millie, Auckland, New Zealand). Cheese blocks (semi-circular with 10 cm diameter  $\times$  2 cm thickness) were then placed in 5 different brine solutions (wt/vol): 10% NaCl (batch A), 7.5% NaCl (batch B), 7.5% (NaCl + KCl, 1:1) (batch C), 5% NaCl (batch D), and 5% (NaCl + KCl, 1:1) (batch E) at 1:5 cheese brine ratio and stored at  $4^{\circ}\text{C}$  for 1 mo. Samples were taken for analysis at 0 (fresh cheese), 10, 20, and 30 d of storage. The experiment was repeated in triplicate.

### Survival of bacteria

For evaluating the survival of bacteria, grated cheese (12.5 g) was blended into 112.5 mL of peptone water (0.15% wt/vol). Serial dilutions were made and each type of bacteria was enumerated on appropriate agar as described below (Tharmaraj and Shah 2003; Dave and Shah 1996; Lapierre and others 1992). Anaerobic conditions, where required, was maintained using anaerobic jars and GasPak anaerobe container system (Becton Dickinson and Co.). Gram staining was carried out and colony morphology was closely monitored to ensure the growth of a pure culture. *Streptococcus thermophilus* MS were enumerated on M17 agar (Becton Dickinson and Co.) and aerobically incubated at  $37^{\circ}\text{C}$  for 24 h. *Lactobacillus delbrueckii* ssp. *bulgaricus* 859 were enumerated using pH-modified (pH 5.2) MRS agar plates and incubated anaerobically at  $40^{\circ}\text{C}$  for 48 h. *Lactobacillus acidophilus* was enumerated on MRS agar supplemented with sorbitol (10% wt/vol; Sigma Aldrich, St. Louis, Mo., U.S.A.) and *Lactobacillus casei* on MRS agar supplemented with vancomycin (2 mL of 0.5 mg/mL vancomycin per liter of media; Sigma Aldrich), and both were incubated anaerobically at  $37^{\circ}\text{C}$  for 48 h. *Bifidobacterium longum* was enumerated using MRS agar supplemented with sodium propionate (3% wt/vol; Sigma Aldrich) and lithium chloride (2% wt/vol; Sigma Aldrich) and the plates were incubated anaerobically at  $40^{\circ}\text{C}$  for 36 to 48 h.

### Determination of sodium, potassium, and calcium contents

Sodium, potassium and calcium contents in cheeses were determined using inductively coupled-plasma optical-emission-spectrometry (PE Optima 8300; PerkinElmer, Mass., U.S.A.; software WinLab32 version 5.1.3.0550) according to Ayyash and others (2012) with some modifications. Cheese (5 g, grated) was digested in a mixture of nitric acid and perchloric acid (5:1; Merck, Darmstadt, Germany) on a hot plate until the mixture was a clear solution. The samples were filtered with a  $0.22\ \mu\text{m}$  Millipore filter and diluted 100 times before analysis. Sodium, potassium, and calcium concentrations in cheese samples were determined using external standard curves of these 3 elements.

### Determination of moisture and pH

Moisture content of cheeses was determined by oven-drying ( $100 \pm 2^{\circ}\text{C}$ ) method (Oksuz and others 2004). pH of cheese samples was measured after blending 10 g of grated cheese in 10 mL distilled water, using a calibrated pH meter (Model 250A, Orion, Boston, Mass., U.S.A.).

### Preparation of water-soluble extracts

Cheese samples (15 g) were homogenized in 15 mL of water with a homogenizer (Ultra Turrax T25, Janke & Kunkel, Staufen im Breisgau, Germany) at  $9500 \times g$  for 2 min. The homogenized slurry was centrifuged for 20 min at  $4000 \times g$  and  $4^{\circ}\text{C}$ . The soluble fraction between the upper layer (fat) and the precipitate (casein) was collected and filtered through Whatman No. 4 filter paper. The extracts were centrifuged at  $4000 \times g$  for 20 min at  $4^{\circ}\text{C}$  and filtered through Whatman glass microfiber filter (GF/C) to obtain a clear supernatant (water-soluble extract, WSE). The water-soluble extracts were concentrated by freeze-drying and the freeze-dried WSEs were stored at  $-20^{\circ}\text{C}$  for determination of amino acids, using reversed-phase high performance liquid chromatography (RP-HPLC), ACE-inhibitory activity, antioxidant activity, and the cell line viability assays.

### Determination of protein content and proteolytic activity

Protein content in water-soluble extracts of cheeses was measured using Bradford method (Bradford 1976) as described by Apostolidis and others (2007). Briefly, 1.5 mL of Bradford reagent was mixed with 0.05 mL WSE and incubated at room temperature (22 °C) for 5 min after which absorbance at 595 nm was recorded using a UV–VIS spectrophotometer (SmartSpec Plus Spectrophotometer, BioRad, Hercules, Calif., U.S.A.). Protein content was calculated using a standard curve plotted using known concentrations of bovine serum albumin (BSA; Sigma Aldrich).

Proteolytic activity of cheese extracts was determined by o-phthalaldehyde (OPA) method of Church and others (1983) with some modifications as described by Mosehishad and others (2013) and Gandhi and Shah (2014). Briefly, 50  $\mu$ L of WSE was added to 1 mL of OPA reagent in a cuvette. The cuvette was inverted twice followed by incubation at room temperature (22 °C) for 2 min. Absorbance was recorded at 340 nm using UV–VIS spectrophotometer (Shimadzu Scientific Instruments, Japan).

### Peptide profiles of cheese extracts using high performance liquid chromatography

An aliquot (40 mg) of freeze-dried WSE was dissolved in 1 mL solvent A (0.1% trifluoroacetic acid (TFA, Sigma-Aldrich), centrifuged (12000  $\times$  g, 10 min) and filtered using 0.22- $\mu$ m-millipore filter. Separation of peptide peaks was performed using RP-HPLC (Model LC-2010A, Shimadzu Corporation, Kyoto, Japan) and Kromasil 5  $\mu$  C-18 column (250 mm  $\times$  4.6 mm; Phenomenex, Calif., U.S.A.). Eluent B was 60% acetonitrile containing 0.05% TFA. A linear gradient was applied from 0% to 80% eluent B over 90 min. Injection volume was 50  $\mu$ L and the separation was conducted at 40 °C at a flow rate of 0.5 mL/min, and detection wavelength was 214 nm.

### Determination of ACE-inhibitory activity

Angiotensin-converting enzyme (ACE)-inhibitory activity of freeze-dried WSE (5 mL freeze-dried WSE in 1 mL distilled water) was determined by the method of Cushman and Cheung (1971) with some modifications as described by Gandhi and Shah (2014). Briefly, an aliquot (200  $\mu$ L) of hippuryl–histidyl–leucine (Sigma Chemical Co.) buffer (5 mM Hip–His–Leu in 0.1 M borate buffer containing 0.3 M NaCl, pH 8.3) was mixed with 60  $\mu$ L of sodium borate buffer and pre-incubated with 20  $\mu$ L of the sample for 5 min at 37 °C. The reaction was initiated by adding 20  $\mu$ L of ACE solution (buffer containing ACE at a concentration of 0.1 unit/mL) followed by incubation at 37 °C for 30 min. Hydrochloric acid (250  $\mu$ L, 1 M) was added to stop the reaction and the solution was then mixed with 1.7 mL of ethyl acetate. The mixture was centrifuged (3000  $\times$  g, 10 min 4 °C) and 1.4 mL of the organic phase (ethyl acetate) was transferred to a fresh test tube. The ethyl acetate was evaporated to dryness on a water bath for 30 min at 100 °C. Hippuric acid residue was dissolved in 1 mL of deionized water, and the absorbance of the solution was recorded using a UV–VIS spectrophotometer (Shimadzu Scientific Instruments, Japan) at 228 nm against water as blank. The ACE-inhibitory activity was calculated as follows:

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

where A is the absorbance in the presence of ACE and without the ACE-inhibitory component, B is the absorbance without ACE and without the ACE-inhibitory component, C is the absorbance

with ACE and the ACE-inhibitory component, and D is the absorbance without ACE and with the ACE-inhibitory component.

### Determination of antioxidant activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of cheese was determined as described by Abadia-Garcia and others (2013). Briefly, 100  $\mu$ L of sample (5 mL freeze-dried WSE in 1 mL distilled water) was mixed with 1.1 mL ethanolic solution of DPPH (0.2 mM). Absorbance was measured at 517 nm using a UV–VIS spectrophotometer (SmartSpec Plus Spectrophotometer, BioRad) after 30 min. The results were expressed as a function of trolox concentration.

### Quantification of amino acids

**Dansylation of free amino acids.** Derivatization of amino acids was performed using dansyl chloride as per Le Vo and others (2012) with some modifications. The reaction mixture consisted of 300  $\mu$ L sample (5 mL freeze-dried WSE dissolved in 1 mL distilled water), 100  $\mu$ L double-distilled water, 200  $\mu$ L 1 M sodium bicarbonate buffer (pH 9.8), 200  $\mu$ L acetonitrile, and 100  $\mu$ L dansyl chloride (40 g/L in acetonitrile). After incubation at 65 °C for 1 h in dark, 100  $\mu$ L of 20% (vol/vol) acetic acid was added to stop the reaction. The reaction mixture was centrifuged at 12000  $\times$  g for 10 min at 25 °C and the supernatant was filtered using a 0.22  $\mu$ m millipore filter and stored at –20 °C for HPLC analysis. Calibration curve for a mixture of 20 standard amino acids was prepared using the same method (Wu and Shah 2015).

**Detection of amino acids by HPLC.** Dansylated amino acids were quantified by RP-HPLC (Model LC-2010A, Shimadzu Corporation, Kyoto, Japan) using a guard column (Phenomenex) and Kromasil 5  $\mu$  100Å C18 column (250 mm  $\times$  4.6 mm; Phenomenex) as described by Wu and Shah (2015). Eluent A was 30 mM ammonium acetate, pH 7.5 and B was acetonitrile. The column was eluted with a linear gradient of 6% to 10% B over 0 to 5 min, 10% to 18% B over 5 to 7 min, 18% to 22% B over 7 to 15 min, 22% to 26% B over 15 to 18.5 min, 26% to 28.5% B over 18.5 to 22.5 min, 28.5% to 30% B over 22.5 to 24 min, 30% to 32% B over 24 to 27.5 min, 32% to 55% B over 27.5 to 40 min, 55% to 50% B over 40 to 45 min, 50% to 6% B over 45 to 55 min and held at 6% B for 5 min. The flow rate of mobile phase was 1 mL/min. Temperature was maintained at 30 °C, injection volume was 20  $\mu$ L and detected wavelength was 275 nm.

### Cell lines

Two cell lines were selected for this study: normal human colon cells, CCD 841 and human colon cancer cells, Caco-2 cells. CCD 841 cells were generously provided by Dr. Tao's lab in The Chinese University of Hong Kong, Hong Kong, and Caco-2 cells were from the American Type Culture Collection (ATCC).

Caco-2 and CCD cells were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM; Gibco BRL, Life Technologies, Grand Island, N.Y., U.S.A.) and Eagle's minimum essential medium (EMEM; ATCC, Manassas, Va., U.S.A.), respectively. Both media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (10000 U/mL) (Gibco BRL). Cells were incubated at 37 °C in an incubator (US Air Flow, NuAire, U.S.A.) with 5% CO<sub>2</sub>. At about 80% confluence, the cells were subcultured by trypsinization using 0.25% trypsin–EDTA (Gibco BRL) and resuspended in fresh media. Caco-2 cells used in this study were between passages 32 and 38, and CCD cells were between passages 11 and 16.



### Cell viability assay

Viability of cells was measured using mitochondrial dehydrogenase activity assay with the substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (van Meerloo and others 2011). Briefly, the cells were subcultured and seeded in 96-well flat bottom plates (Nunc, Thermo Scientific, Suzhou, China) at  $2 \times 10^4$  cells/mL and allowed to grow for 48 h. The cells were then washed with sterile PBS and cultured with antibiotic free media supplemented with varying concentrations (0 to 2000  $\mu\text{g/mL}$ ) of freeze-dried water-soluble extracts (WSE) of cheeses (batch A and batch C). The WSE was freshly prepared by dissolving freeze-dried WSE in media in appropriate dilutions. An aliquot (100  $\mu\text{L}$ ) of filter sterilized sample (pure media with WSE) or control (pure media with no WSE) was added to each well and incubated for 24 h in the  $\text{CO}_2$  incubator. The culture media was then inoculated with 10  $\mu\text{L}$  MTT reagent (0.5 mg/mL; Sigma) under dim light and the plates were incubated for 4 h at 37 in dark. The crystal product formed was dissolved using 100  $\mu\text{L}$  acidified isopropanol and absorbance was recorded at 595 nm using iMark microplate reader (BioRad).

### Statistical analysis

All experiments were replicated thrice and all analyses were carried out in duplicate. Data obtained were analyzed using one-way analysis of variance (ANOVA) at 95% level of significance using SPSS statistics software v 20.0 (IBM Corp, Armonk, N.Y., U.S.A.). Tukey's *post hoc* analysis was performed to further investigate the difference in the means of different cheeses at same storage period. Pearson correlation coefficients were determined in the correlation analyses.

## Results and Discussion

### Survival of bacteria

Survival of starter and probiotic bacteria was monitored at every 10 d during storage for 1 mo (Figure 1). No significant differences

( $P > 0.05$ ) in colony forming units (CFU) of probiotic bacteria were observed at day 10 among different batches of cheese compared to the control (batch A; 10% NaCl). However, after 20 and 30 d of storage significant differences were observed between different batches of cheese in survival of all bacteria. In case of *S. thermophilus* significant ( $P < 0.05$ ) difference was observed after 10 d of storage in batch C and E as compared with the control. In general, increased concentrations of KCl and reduced concentrations of NaCl promoted the growth of starter and probiotic bacteria at any given storage period. The growth of all bacteria was significantly higher in batch C, D, and E, as compared to the control after 30 d. This could be due to either the binding of less sodium ions or more potassium ions to bacterial membrane phospholipids; this binding is weaker than that of sodium ions, thus causing lesser damage to bacterial membrane (Gurtovenko and Vattulainen 2008).

### Moisture content and pH

Moisture content of Akawi cheese was not significantly ( $P > 0.05$ ) affected by varying NaCl/KCl concentrations up to 20 d of storage (Table 1), although an increasing trend was observed. Similar trend was observed in Cheddar cheese salted with varying concentrations of NaCl/KCl (Hystead and others 2013). However, after 30 d, significant ( $P < 0.05$ ) difference in batch B and D, which contain only reduced NaCl, was observed. Akawi cheese, like most Middle Eastern brined cheeses is characterized by acidic taste (Tamime 2008). The pH of different batches of cheese was not significantly ( $P > 0.05$ ) affected by salt concentrations during 30 d storage (Table 1). The moisture content and pH range are different than those reported by Ayyash and others (2012) which may be attributed to several factors such as difference in milk, type of chymosin (Chy Max plus was used in this study), differences in bacterial strains, and addition of another bacterium (*B. longum*). The pH increased slightly ( $P > 0.05$ ) during storage for batch C cheese that was stored in brine solutions containing KCl. This

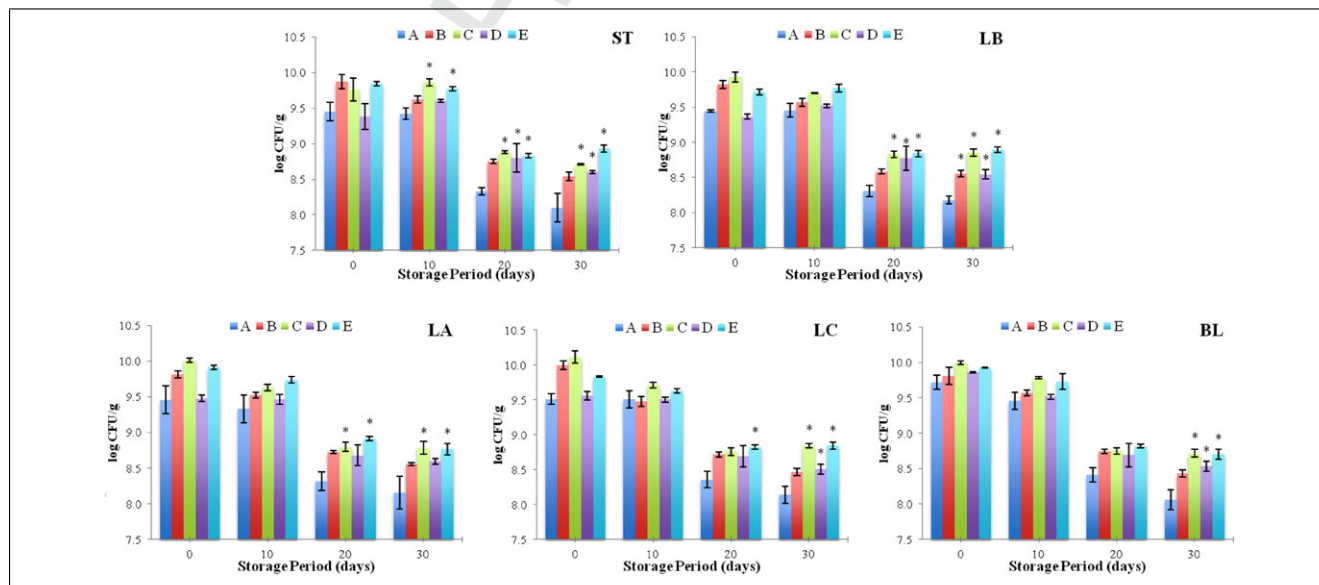


Figure 1—Survival of starter and probiotic bacteria in cheese as affected by NaCl reduction and substitution with KCl during 30 d storage at 4 °C. <sup>a</sup>Brine Solutions: 10% NaCl (A); 7.5% NaCl (B); 7.5% NaCl+KCl (1:1) (C); 5% NaCl (D); 5% NaCl+KCl (1:1) (E). \*Means within a storage period are significantly different ( $P < 0.05$ ) from the control (batch A). Values are means  $\pm$  SE ( $n \geq 3$ ). ST, *S. thermophilus*; LB, *L. delbrueckii* ssp. *bulgaricus*; LA, *L. acidophilus*; LC, *L. casei*; BL, *B. longum*.

**Table 1—Moisture content and pH of cheeses as affected by NaCl reduction and substitution with KCl during 30 d storage at 4 °C.**

Storage time (d)	Brine solution <sup>a</sup>	Moisture content (%)	Ph
0	A	54.50 ± 1.33	5.28 ± 0.02
	B	53.44 ± 1.85	5.03 ± 0.04
	C	58.63 ± 0.38	4.95 ± 0.03
	D	55.67 ± 0.92	5.12 ± 0.01
	E	58.15 ± 2.25	5.00 ± 0.03
10	A	56.79 ± 1.39	5.15 ± 0.06
	B	60.47 ± 4.49	5.06 ± 0.02
	C	62.99 ± 1.89	4.95 ± 0.01
	D	63.17 ± 1.80	5.08 ± 0.01
	E	64.36 ± 0.85	5.14 ± 0.02
20	A	57.26 ± 1.23	5.30 ± 0.03
	B	65.58 ± 1.19	5.04 ± 0.01 <sup>b</sup>
	C	63.17 ± 1.76	4.86 ± 0.01 <sup>b</sup>
	D	63.27 ± 3.65	4.79 ± 0.03 <sup>b</sup>
	E	60.74 ± 2.16	5.12 ± 0.04
30	A	57.22 ± 1.53	5.42 ± 0.32
	B	65.04 ± 1.10 <sup>b</sup>	4.98 ± 0.05
	C	56.91 ± 0.60	5.52 ± 0.03
	D	61.74 ± 0.38 <sup>b</sup>	4.83 ± 0.05
	E	57.53 ± 0.74	4.89 ± 0.00

Values are means ± SE (*n* ≥ 3).

<sup>a</sup>Brine solutions: 10 % NaCl (A); 7.5 % NaCl (B); 7.5 % NaCl+KCl (1:1) (C); 5 % NaCl (D); 5 % NaCl+KCl (1:1) (E).

<sup>b</sup>Means within a column and within each day are significantly different (*P* < 0.05) as compared to control (batch A).

difference may be due to higher pH of KCl solution as compared to NaCl solution (Katsiari and others 1997).

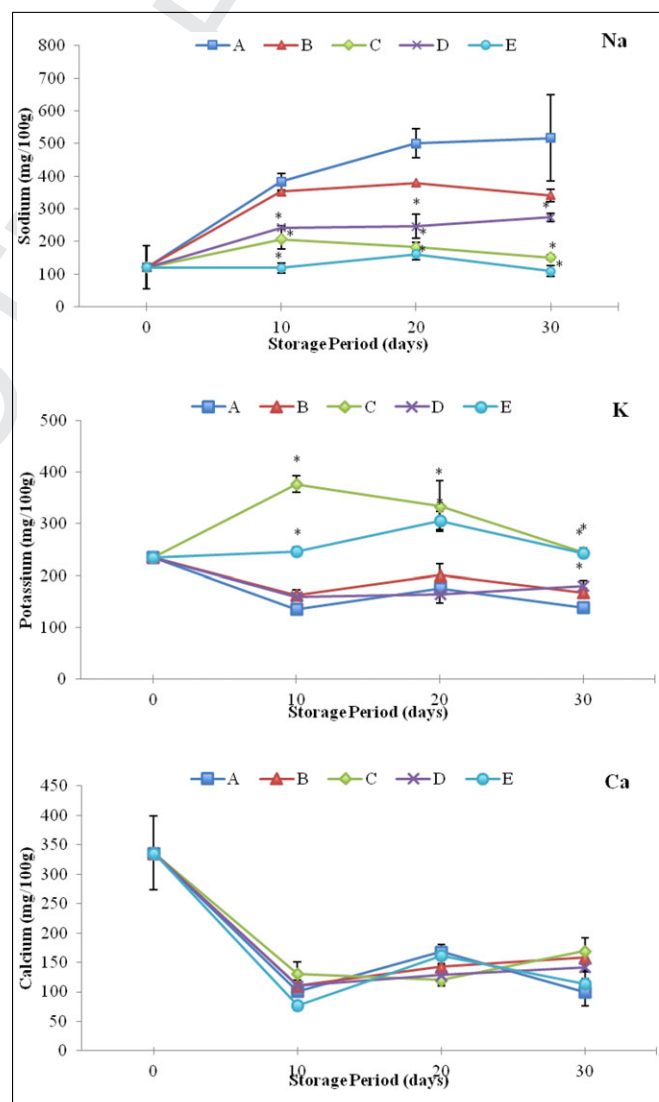
### Sodium, potassium and calcium contents

Sodium, potassium and calcium contents as determined by optical emission spectrometry are shown in Figure 2. Sodium and potassium contents of cheeses were found to be in accordance with the amount of sodium and potassium, respectively, in the brine solution, that is sodium content in A was highest followed by B, D, C, and E, respectively. The sodium content in cheese was significantly (*P* < 0.05) lower in batches C, D, and E, as compared to the control after 10 d of storage. After 10 d of storage in brine solutions, the highest sodium content was observed in the control (batch A) 392 mg per 100 g cheese, as compared to batch E which was 118 mg per 100 g. Potassium content was found to be in agreement with the addition of potassium in the brine solution. Significant (*P* > 0.05) increase was observed in the potassium levels in batch C and E after 10 d of storage in brine containing KCl. A decrease in the calcium content in all cheese types was observed which might be due to transfer of calcium ions from cheese matrix into the brine solution during storage. However, no significant (*P* > 0.05) differences were observed in the calcium content within different experimental cheeses beyond 10 d of storage. The decrease in calcium content during brining is attributed to the sodium–calcium interchange or potassium–calcium interchange (Guinee and Fox 1993). It has been reported that addition of 5% sodium chloride in cheese matrix increases the calcium content in the serum (Creamer 1985).

### Protein Content, Proteolytic Activity, and Peptide Profile

Protein content in water-soluble extracts of cheese as determined by Bradford method is shown in Figure 3(A). A reduction in protein content in WSE of most types of cheeses was observed after 10 d of storage. This reduction in initial 10 d of storage could

possibly be due to the migration of solubilized casein and serum proteins in fresh cheese into the brine solution (Katsiari and others 2000). Another possible cause of protein reduction in WSE could be due to movement of soluble minerals in the cheese during brining, thereby affecting the protein conformations in cheese matrix. During brining in salt, the sodium and the chloride ions migrate from the brine into the cheese matrix due to the osmotic pressure differences in cheese and brine. As a result of this, and to restore osmotic pressure equilibrium, the water (from cheese) containing soluble acids and minerals diffuse out with a flux which is 2 times more than that of NaCl (Guinee and Fox 1993). These dynamic and complex transport systems cause several changes in the composition and texture of cheese during ripening based on several variables (such as brine temperature, cheese to brine ratio, and so on). A decrease in the protein levels during ripening of Turkish White cheese was attributed to diffusion of proteolytic



**Figure 2—Sodium (Na), potassium (K) and calcium (Ca) contents of cheeses as affected by NaCl reduction and substitution with KCl during 30 d storage at 4 °C.** <sup>a</sup>Brine Solutions: 10% NaCl (A); 7.5% NaCl (B); 7.5% NaCl+KCl (1:1) (C); 5% NaCl (D); 5% NaCl+KCl (1:1) (E). \*Means within a storage period are significantly different (*P* < 0.05) from the control (batch A). Values are means ± SE (*n* ≥ 3).

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2  
3 products into the brine (Hayaloglu and others 2002), as a result of  
4 which the amount of total nitrogen in the brine increases during  
5 ripening (Ehsani and others 1999).

6 The proteolytic activity during storage plays an important role  
7 in release of flavor compounds and amino acids. In this study,  
8 the properties of the water-soluble cheese extract, which is rich  
9 in peptides and amino acids, were evaluated. Cheeses brined in  
10 varying salt (NaCl/KCl) concentrations would impact the proteolysis  
11 in cheese and thus possibly the amino acids and the activity  
12 of the peptides released. It was therefore critical to evaluate the  
13 bioactivities (such as antioxidant, ACE-inhibitory, and colon cell  
14 viability) in the water-soluble extracts of these cheeses. The increased  
15 proteolytic activity (Figure 3B) observed in Akawi cheese  
16 WSE, particularly in those brined with lower NaCl concentration,  
17 contributed to enhanced production of amino acids in the  
18 product (Table 2). The changes in the overall peptide profile of  
19 WSE of cheese indicate increased presence of peptides in cheeses  
20 brined in lower salt concentration (7.5% and 5%) as compared to  
21 those brined in higher total salt concentrations (10%). The peptide  
22 profile (Figure 3C) of all stored cheeses showed increased proteolysis  
23 as compared to the profile of fresh cheese, which indicated  
24 that there was increased proteolysis in cheese during storage. The  
25 peak intensities of the peaks in highlighted regions were the most  
26 significantly affected by varying salt concentrations of the brine  
27 solutions. Batch C (segments S1 to S3, Figure 3C) shows most  
28 changes in the number and intensity of peaks as compared to the  
29 control, batch A. An obvious increase in the peak at the retention  
30 time of 28, 64, 66, 68, and 78 min was observed in batch C as

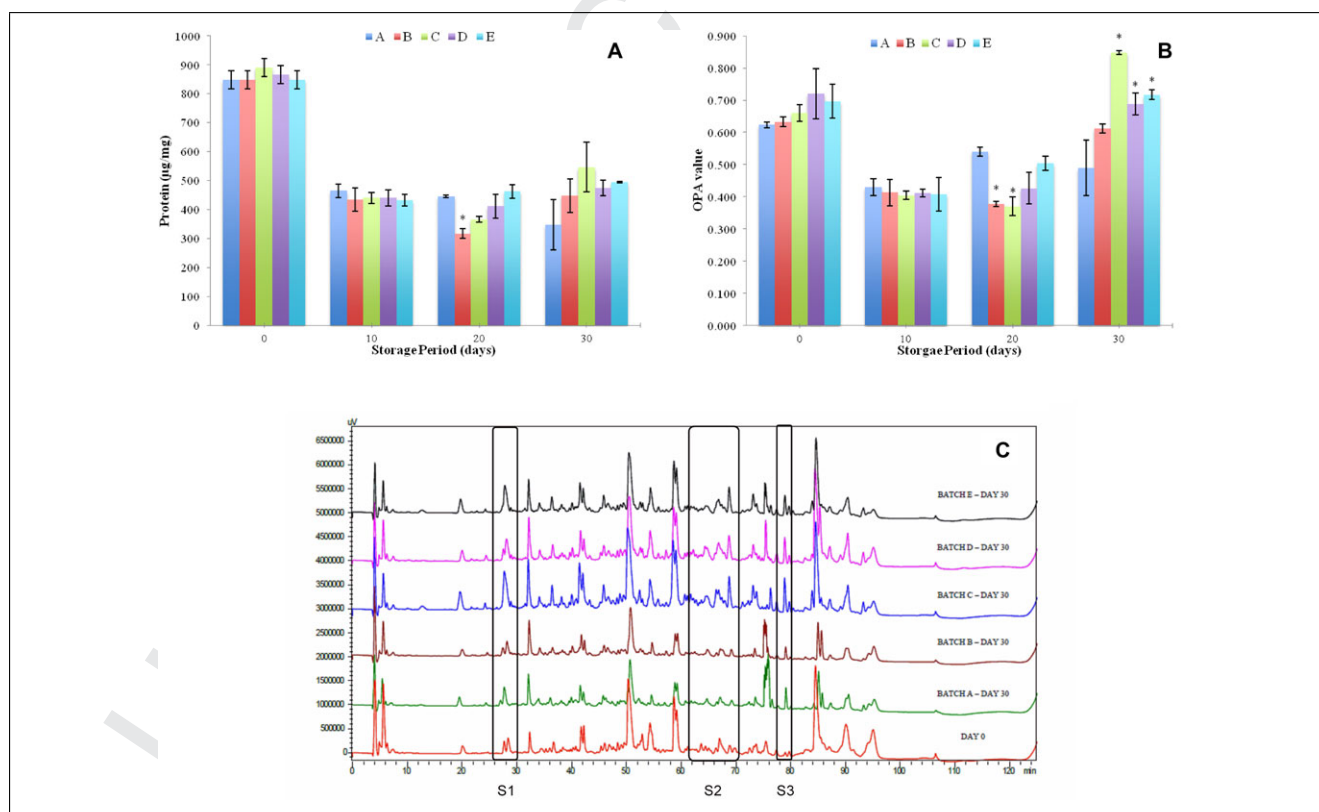
compared with the batch A (control). These changes in peptides  
are indicative of increased proteolysis in batch C, which was brined  
with 7.5% NaCl:KCl (1:1).

### ACE-inhibitory activity

ACE-inhibitory peptides are biologically active peptides that  
possess antihypertensive properties and have been detected in  
fermented milk products, released by bacterial proteinases and  
peptidases during fermentation. In this study, the ACE-inhibitory  
activity of freeze-dried WSE of Akawi cheese was monitored  
in different batches during storage (Figure 4). In general, ACE-  
inhibitory activity was found to be higher in batch D and E,  
which had the lowest amount of salt concentration. Although at a  
given storage time no significant differences ( $P > 0.05$ ) were  
observed in different batches of cheese as compared with the control,  
highest inhibitory activity (92.56%) was observed in batch E after  
30-d storage. No significant changes in ACE-inhibitory activity,  
in cheeses brined with varying KCl concentrations, were observed  
by (Ayyash and others 2012). This trend could possibly be due to  
slow proteolysis continuing during the prolonged storage. These  
findings indicate that reduction and substitution of NaCl with  
KCl may not be affecting the release of ACE-inhibitory peptides  
in Akawi cheese.

### Antioxidant activity

Antioxidant activity of freeze-dried WSE of Akawi cheese was  
estimated by DPPH radical scavenging ability (Figure 5). The  
antioxidant activity (expressed as  $\mu\text{M}$  equivalence of trolox) was not



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Figure 3—Protein content (A), proteolytic activity (B) and peptide profile (C) of WSE of cheeses as affected by NaCl reduction and substitution with KCl during 30 d storage at 4 °C. <sup>a</sup>Brine Solutions: 10% NaCl (A); 7.5% NaCl (B); 7.5% NaCl+KCl (1:1) (C); 5% NaCl (D); 5% NaCl+KCl (1:1) (E). \*Means within a storage period are significantly different ( $P < 0.05$ ) from the control (batch A). Values are means  $\pm$  SE ( $n \geq 3$ ). WSE: water soluble extract; S1–S3, segments of chromatogram with altered peaks.

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**Table 2—Amino acid content in cheeses as affected by NaCl reduction and substitution with KCl during 30 d storage at 4 °C.**

	Batch A <sup>a</sup>			Batch B <sup>a</sup>			Batch C <sup>a</sup>			Batch D <sup>a</sup>			Batch E <sup>a</sup>		
	10	20	30	10	20	30	10	20	30	10	20	30	10	20	30
Aspartic acid	0.140	0.123	0.099	0.178	0.153	0.164	0.141	0.132	0.162	0.241 <sup>b</sup>	0.127 <sup>c</sup>	0.103 <sup>c</sup>	0.121	0.129	0.118
Glutamic acid	0.251	0.196	0.185	0.388	0.287	0.344 <sup>b</sup>	0.290	0.238	0.608 <sup>b,c</sup>	0.236	0.197	0.158	0.194	0.220	0.341 <sup>b,c</sup>
Asparagine	0.063	0.079	0.032	0.059	0.041 <sup>b</sup>	0.060	0.058	0.045	0.156 <sup>b,c</sup>	0.053	0.048	0.048	0.038	0.063	0.060
Glutamine	0.131	0.145	0.123	0.136	0.119	0.223	0.105	0.102	0.549 <sup>b,c</sup>	0.110	0.094	0.164	0.080	0.139	0.161
Arginine + Serine	0.054	0.061	0.057	0.035	0.029	0.062	0.039	0.038	0.219 <sup>b,c</sup>	0.042	0.040	0.065	0.050	0.068	0.111 <sup>b,c</sup>
Threonine	0.021	0.028	0.030	0.026	0.027	0.048	0.028	0.028	0.121 <sup>b,c</sup>	0.060 <sup>b</sup>	0.033 <sup>c</sup>	0.037	0.030	0.039	0.046
Glycine	0.061	0.040	0.051	0.067	0.058	0.073	0.058	0.058	0.174 <sup>b,c</sup>	0.070	0.048	0.054	0.048	0.051	0.067
Alanine	0.027	0.027	0.050	0.059	0.055	0.096 <sup>b,c</sup>	0.064 <sup>b</sup>	0.064 <sup>b</sup>	0.132 <sup>b,c</sup>	0.099 <sup>b</sup>	0.066	0.071	0.057 <sup>b</sup>	0.064 <sup>b</sup>	0.077
Proline	0.337	0.325	0.193 <sup>c</sup>	0.392	0.381	0.420 <sup>b</sup>	0.344	0.343	0.443 <sup>b</sup>	0.396	0.331	0.165 <sup>c</sup>	0.253	0.310	0.196
Valine	0.085	0.096	0.074	0.070	0.099	0.122 <sup>b,c</sup>	0.097	0.095	0.277 <sup>b,c</sup>	0.075	0.094	0.096	0.087	0.100	0.128 <sup>b,c</sup>
Methionine	0.138	0.192	0.144	0.094	0.114 <sup>b</sup>	0.119	0.096	0.108 <sup>b</sup>	0.159	0.081	0.082 <sup>b</sup>	0.093	0.062	0.081 <sup>b</sup>	0.073
Isoleucine	0.051	0.044	0.044	0.047	0.068	0.097 <sup>b,c</sup>	0.057	0.055	0.111 <sup>b,c</sup>	0.077 <sup>b</sup>	0.065	0.072 <sup>b</sup>	0.051	0.073 <sup>b</sup>	0.073 <sup>b</sup>
Leucine	0.104	0.115	0.107	0.091	0.130	0.196 <sup>b,c</sup>	0.125	0.126	0.510 <sup>b,c</sup>	0.173	0.125	0.171	0.116	0.147	0.250 <sup>b,c</sup>
Tryptophan	0.059	0.070	0.060	0.060	0.071	0.087	0.062	0.066	0.165 <sup>b,c</sup>	0.062	0.063	0.082	0.057	0.074	0.087
Phenylalanine	0.111	0.133	0.150	0.087	0.141	0.261 <sup>b,c</sup>	0.126	0.128	0.671 <sup>b,c</sup>	0.163	0.133	0.174	0.110	0.196 <sup>c</sup>	0.403 <sup>b,c</sup>
Cysteine	0.069	0.103	0.060	0.091	0.100	0.102	0.073	0.076	0.252 <sup>b,c</sup>	0.078	0.063	0.080	0.057	0.085	0.096
Lysine+Histidine	0.038	0.054	0.034	0.026	0.054	0.082 <sup>b,c</sup>	0.044	0.063	0.098 <sup>b,c</sup>	0.073	0.081	0.082 <sup>b</sup>	0.086 <sup>b</sup>	0.091	0.092 <sup>b</sup>
Tyrosine	0.066	0.081	0.070	0.048	0.077	0.091	0.077	0.086	0.144 <sup>b,c</sup>	0.066	0.091	0.106	0.087	0.093	0.183 <sup>b,c</sup>

Values are means ± SE (n ≥ 3) and are expressed as g/L.

<sup>a</sup>Brine solutions: 10% NaCl (A); 7.5% NaCl (B); 7.5% NaCl+KCl (1:1) (C); 5% NaCl (D); 5% NaCl+KCl (1:1) (E).

<sup>b</sup>Means are significantly different (P < 0.05) as compared to the respective day of control (batch A).

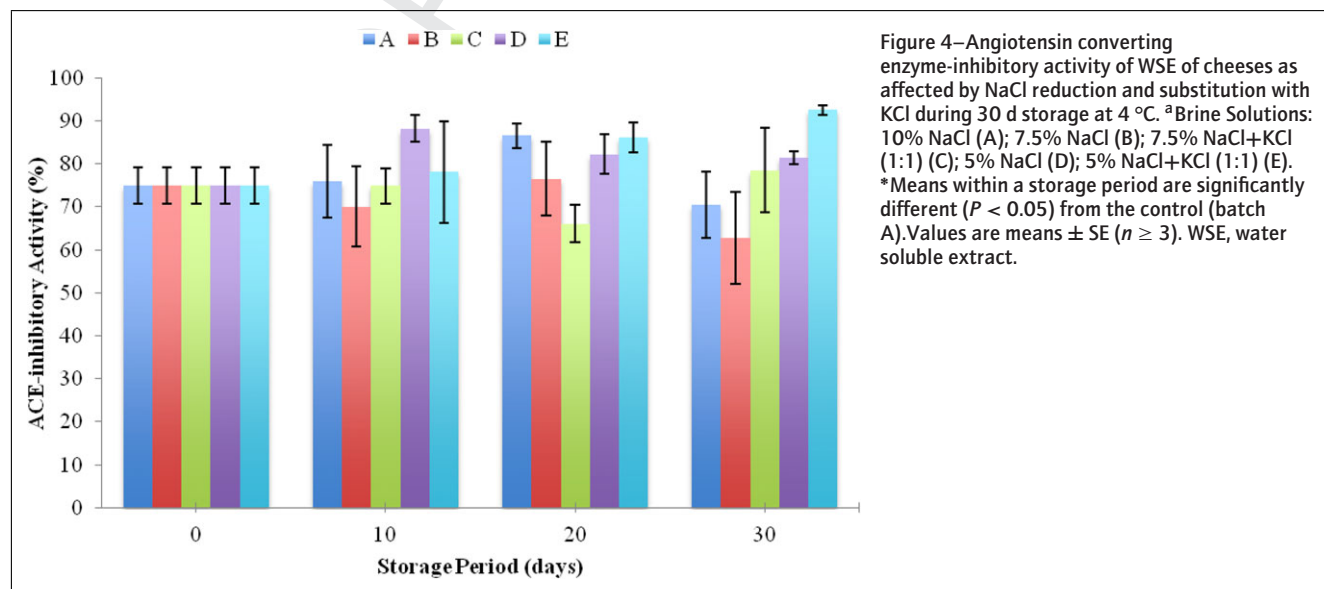
<sup>c</sup>Means within a batch are significantly different (P < 0.05) as compared to day 10 of the same batch.

significantly (P > 0.05) affected by the concentration of NaCl/KCl in brine solutions. A decline in μM equivalence of trolox was observed within the first 10 d of storage beyond which the activity was stable. Similar trend was observed in all 5 batches of cheese during storage. The initial decrease in the activity may be possibly due to the breakdown and utilization of antioxidant peptides released during manufacture of Akawi cheese. Among the different experimental cheese, batch E (5% NaCl+KCl, 1:1) showed the least trolox equivalence (197 μM trolox), whereas highest was observed in batch C (288 μM trolox; 7.5% NaCl+KCl, 1:1). Also, least decline during storage was observed in batch C, which indicated that 50% substitution of NaCl with KCl at 7.5% total salt appears to be the optimal brine solution for maintaining the antioxidant activity of Akawi cheese during storage. Furthermore, no strong correlation was found between the antioxidant capacity

and proteolytic activity as observed by Pearson correlation coefficient (0.0135). These findings indicate that antioxidant peptides (as a result of altered proteolysis) largely remain unaffected by the variations of salt in brine solutions.

### Release of amino acids

The effect of different NaCl/KCl concentrations in brine solutions on the concentration of twenty standard amino acids during storage is shown in Table 2. During separation of amino acids by RP-HPLC, the peaks of arginine and serine, and those of lysine and histidine could not be completely separated, and the data shown included a total of the 2 in each case. The concentrations of amino acids in each batch during storage were compared to the control batch and statistically analyzed. Statistical analysis of the effect of storage within a batch was also evaluated. The trends



**Figure 4—Angiotensin converting enzyme-inhibitory activity of WSE of cheeses as affected by NaCl reduction and substitution with KCl during 30 d storage at 4 °C.** <sup>a</sup>Brine Solutions: 10% NaCl (A); 7.5% NaCl (B); 7.5% NaCl+KCl (1:1) (C); 5% NaCl (D); 5% NaCl+KCl (1:1) (E). \*Means within a storage period are significantly different (P < 0.05) from the control (batch A). Values are means ± SE (n ≥ 3). WSE, water soluble extract.

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in changes of amino acid content are shown in the heat map (Figure S1), with red color depicting the lower concentrations and green depicting the higher concentrations. Slight changes were observed in the amino acid profiles of cheeses stored in low salt brine solutions (5%), but significant changes were observed in cheeses stored in 7.5% brine solution. In batch A (10% NaCl), there was no significant difference ( $P > 0.05$ ) on concentrations of most of the amino acids during storage. In cheese stored in 7.5% brine solution (batches B and C), there was significant ( $P < 0.05$ ) increase in concentration of most of the amino acids after 30 d. This could be attributed to an increased proteolytic activity in cheeses stored at 7.5% brine solution, which appeared to enhance the amino acid release. A strong correlation was observed between proteolytic activity and total essential amino acids (EAA) in cheese extract as observed by Pearson correlation coefficient analyses. The correlation coefficient was strongest for batch E (0.998) and batch C (0.996). Nevertheless, a positive correlation was also observed in all other batches of cheese (Pearson coefficient for batches A, B and D was 0.956, 0.855, and 0.720, respectively). The EAA in cheese samples from batch D and E did not increase significantly ( $P > 0.05$ ) during storage. Among EAA, the concentrations of valine, leucine, tryptophan, and phenylalanine increased during storage in most cheeses. This increase correlated to the increased proteolytic activity as observed during storage (Figure 3B). Certain flavor compounds originating from phenylalanine and tryptophan have been detected in cheese (Adda and others 1982) as well as in model systems (Jollivet and others 1994). Release of amino acids plays a significant role in determining the production of flavor compounds in cheese. Furthermore, the increased concentration of amino acids, in particular the essential amino acids, in cheese also contributes to enhanced properties, especially the flavor, of the product. Overall, the study revealed that reduction of NaCl to 5% (batches D and E) does not contribute to significant increase in amino acids during storage. On the contrary, reduction up to 7.5% brine solution (batch B) and 50% substitution with KCl (batch C) aided in enhanced concentrations of amino acids during storage in Akawi cheese.

### Effect of water-soluble extract on growth of colon cells

The effect of WSE on the growth of human colon cells and human colon cancer cells was investigated (Figure 6). One of the most reliable and commonly used methods for determining cell viability is based on reduction of tetrazolium salts such as MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide). The yellow tetrazolium salt, MTT, is reduced by the action of dehydrogenase enzymes in metabolically active cells to generate reducing equivalents such as NADH and NADPH. This reduction of MTT results in formation of a purple colored formazan, which can be solubilized and quantified using a spectrophotometer. The Caco-2 cells are heterogeneous human epithelial colorectal adenocarcinoma cells, and CCD 841 is a cell line of human epithelial colon cells, and both cell lines are adherent in nature.

The WSE of cheese brined in 7.5% NaCl+KCl (1:1) solution was selected for the cell line study as it was found to be the optimum concentration as mentioned previously, and the effects were compared to those of the WSE of cheese brined in 10% NaCl solution (control). Viability of CCD cells was significantly ( $P < 0.05$ ) increased at lower concentrations (50 to 100  $\mu\text{g}/\text{mL}$ ) of WSE from batch C. There was no significant change in CCD cells at concentrations ranging from 250 to 750  $\mu\text{g}/\text{mL}$ , beyond which there was a significant ( $P < 0.05$ ) decline in cell viability, which is indicative of the cytotoxic effects of WSE from batch C at high doses (1000 to 2000  $\mu\text{g}/\text{mL}$ ). The WSE from control batch (batch A), showed a similar trend, however, the cell viability was higher in those supplemented with WSE from batch C. The lowest cell viability was 88% (compared to the control) at 2000  $\mu\text{g}/\text{mL}$  of WSE from batch C. The WSE from both batches, A and C, had an inhibitory effect on the viability of Caco-2 cells. The viability was significantly reduced at concentrations higher than 75  $\mu\text{g}/\text{mL}$  WSE. At highest concentration (2000  $\mu\text{g}/\text{mL}$ ) evaluated, the cell viability was approximately 65% of the cell viability in control (no WSE treatment). Enzymatic hydrolysates from casein have shown inhibitory effect on growth of Caco-2 cells (Phelan and others 2010) at high concentrations (5% to 10% vol/vol). Our results clearly indicated that the WSE from Akawi cheese may

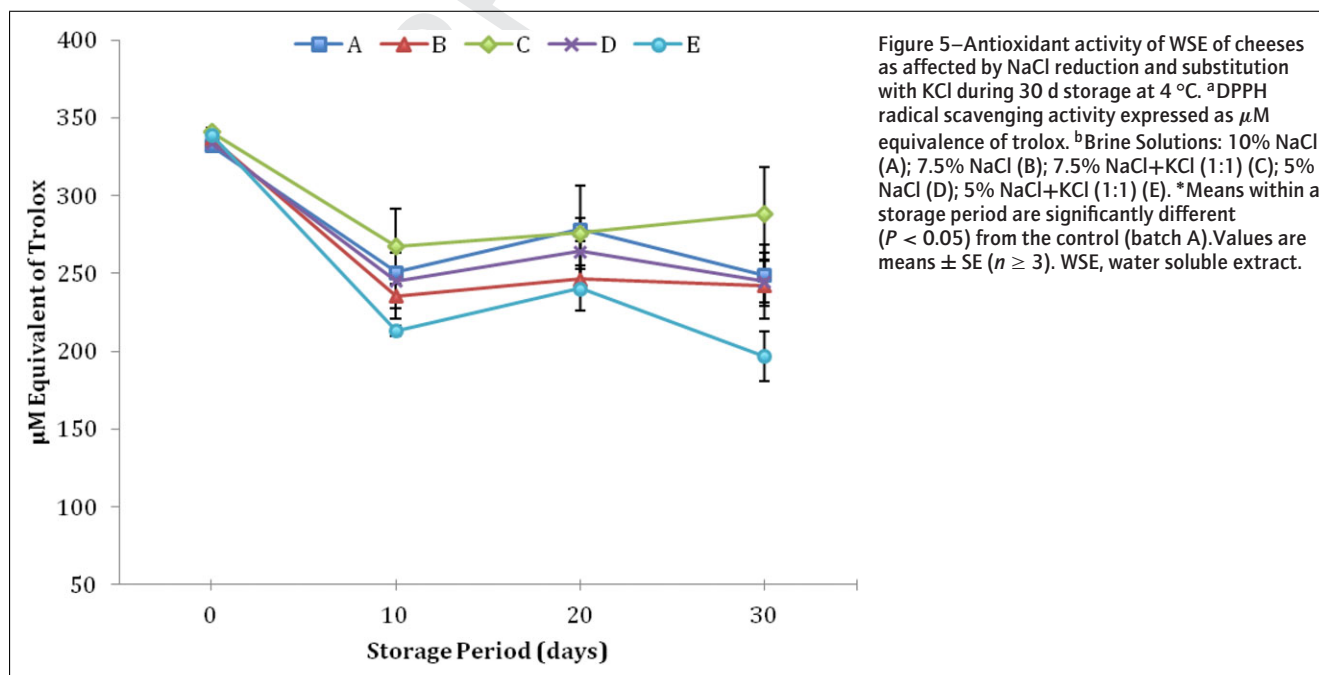


Figure 5—Antioxidant activity of WSE of cheeses as affected by NaCl reduction and substitution with KCl during 30 d storage at 4 °C. <sup>a</sup>DPPH radical scavenging activity expressed as  $\mu\text{M}$  equivalence of trolox. <sup>b</sup>Brine Solutions: 10% NaCl (A); 7.5% NaCl (B); 7.5% NaCl+KCl (1:1) (C); 5% NaCl (D); 5% NaCl+KCl (1:1) (E). \*Means within a storage period are significantly different ( $P < 0.05$ ) from the control (batch A). Values are means  $\pm$  SE ( $n \geq 3$ ). WSE, water soluble extract.



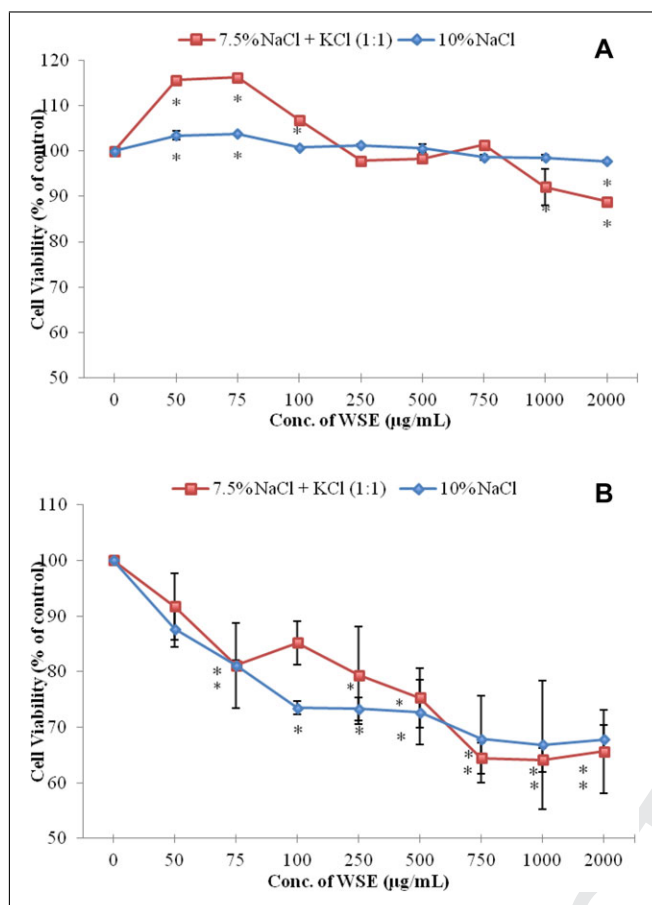


Figure 6–Cell viability of human colon cells, CCD (A) and human colon cancer cells, Caco-2 (B) as affected by WSE of cheeses brined in 7.5 % NaCl+KCl (1:1) and 10 % NaCl (control). Cell viability was expressed as a percentage compared with the control (no treatment with WSE; 0 µg/mL). \*Means within a storage period are significantly different ( $P < 0.05$ ) from the control (no treatment with WSE; 0 µg/mL). Values are means  $\pm$  SE ( $n \geq 3$ ). WSE, water soluble extract.

have the potential to retard the growth of colon cancer cells even at low concentrations. Cytochemical studies have revealed apoptosis inducing ability of casein-derived peptides. Cytotoxic effect of individual casein fractions, and of mixture of amino acids and peptides from casein hydrolysates against cancer cells has also been reported (Chan-Remillard and Ozimek 2008; Zhao and others 2014). Some studies have also shown that casein derived peptides such as  $\beta$ -casein f(60 to 66),  $\beta$ -casein f(1 to 25),  $\kappa$ -casein f(17 to 21),  $\alpha$ -casein f(90 to 96) possessed the ability to induce apoptosis. The most probable mechanism of apoptotic activity is through involvement of opioid receptors (Lopez-Exposito and Recio 2008).

## Conclusion

The effect of NaCl reduction to 7.5% and 50% substitution of NaCl with KCl at reduced concentration (7.5%) in Akawi cheese affected the survival of the bacteria and their proteolytic activity. Increased concentration of several amino acids observed in cheeses stored in reduced NaCl concentrations (7.5%) for 1 mo was strongly correlated to the proteolytic activity. However, there was no significant effect on the activities of potential bioactive peptides released during storage in varying NaCl concentrations. The study revealed that brine concentration and composition (NaCl/KCl) affected the proteolytic activity and in turn the release of amino acids and the activities of peptides released, and that a reduced

concentration (7.5%) may improve the functional properties of cheese. The cheese extracts also showed the potential to enhance the growth of human colon cells and to retard the growth of human colon cancer cell. This study paves way for further research on gut-health promoting effects of cheese extracts.

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## Supporting Information

Additional Supporting Information may be found in the online  
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**Figure S1.** Heat map showing change in amino acid during  
storage in cheeses brined 691 with varying salt (NaCl/KCl)  
concentrations.

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3 **Queries**  
4

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