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## ORIGINAL ARTICLE

# Comparative antioxidant activity, proteolysis and *in vitro* $\alpha$ -amylase and $\alpha$ -glucosidase inhibition of *Allium sativum*-yogurts made from cow and camel milk



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Received 22 January 2011; accepted 24 September 2011

Available online 1 October 2011

## KEYWORDS

Yogurt;  
*Allium sativum*;  
Total phenolic content;  
Antioxidant;  
Proteolysis

**Abstract** The presence of extract of plant with medicinal properties during milk fermentation could enhance the therapeutical values of yogurt. In the present study, the effects of *Allium sativum* on the changes in post-acidification, total phenolic content (TPC), proteolysis by *o*-phthaldialdehyde (OPA) assay, antioxidant activity by (1,1-diphenyl-2-picrylhydrazyl radical (DPPH) inhibition) and capacity to inhibit *in vitro*  $\alpha$ -amylase and  $\alpha$ -glucosidase activities in cow or camel milk yogurt (MY) during 21 day refrigerated storage were investigated. The presence of *A. sativum* enhanced more pH reduction for camel-MY than for cow-MY compared to their respective controls during storage. The reverse was true for total titratable acid. TPC in camel-MY was higher ( $p < 0.05$ ) than that in cow-MY. The presence of *A. sativum* in cow- and camel-MYs elevated ( $p < 0.05$ ) the TPC, but these changed little during storage. Antioxidant activities (18–38% DPPH inhibition) were not different in both types of yogurts, either in the absence or in the presence of *A. sativum*. However, camel-MY had an increase ( $p < 0.05$ ) in antioxidant activities (49–65%) during 7–21 days of storage. OPA values on day 0 was higher for camel-MY ( $368.2 \pm 14.8$  mg/g) than for cow-MY ( $80.1 \pm 3.2$  mg/g). The presence of *A. sativum* increased OPA values more for cow-MY than for camel-MY (3.0- and 1.3-folds, respectively). Higher inhibition ( $p < 0.05$ ) of  $\alpha$ -amylase

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by camel-MY compared to cow-MY occurred whereas  $\alpha$ -glucosidase inhibition by cow-MY reduced ( $p < 0.05$ ) as a result of refrigeration greater than 7 days. In general, the addition of *A. sativum* caused more antioxidant activities, proteolysis and enzymes ( $\alpha$ -amylase and  $\alpha$ -glucosidase) inhibition in camel-MY than in cow-MY.

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## 1. Introduction

Drinking camel milk for the purpose of treating diabetes mellitus is a common practice in Africa, Asia and the Middle East (Yagil et al., 1994). Camel milk has special properties not found in the milk of other mammals (Yagil, 1987) apart from the essential nutrients available from cow milk (Yagil, 1982). Camel milk also offers several unique health benefits attributed to the presence of high concentration of insulin/insulin like protein, immunoglobulin, lactoferrin, lactoperoxidase and peptidoglycan recognition protein (Wangoh, 1993). The non-coagulum formation of camel milk in acidic environment made this milk readily available for absorption in the intestine (Wangoh, 1993). Furthermore, camel milk has no allergic properties which made it suitable to be consumed by lactase deficient persons and those with weak immune systems (Al-Attas, 2008).

Yogurt is one of the most popular fermented foods and traditionally consumed for a long time in many countries (Nakasaka et al., 2008). It is formed during the slow lactic fermentation of milk lactose by the thermophilic lactic acid bacteria.

Cow milk yogurt contributes to most of the currently available yogurt because of the highly successful commercialization of cow milk. The presence of  $\beta$ -immunoglobulin ( $\beta$ -lg) in cow milk was considered as one of the major protein antigens responsible for allergic reaction in infants (Lara-Villoslada et al., 2005; El-Agamy, 2007). Camel milk is free of  $\beta$ -lg (Kappeler, 1998) and in this regard consuming camel milk should cause far less hypersensitivity reactions than consuming cow milk.

Medicinal plants rich in natural anti-oxidants and phenolics are increasingly used in food manufacturing because they provide valuable nutritional and therapeutic properties and retard oxidative degradation of lipids. Additionally, the quality and nutritional values of foods regarded as functional such as herbal-yogurt may also be improved (Shori and Baba, 2011). The development of functional food via biotechnology is therefore important to create products enrichment with therapeutic benefits to address today's modern ways of life and unhealthy diets' effect on the consumers' health. The consumption of garlic for instance was shown to reverse hyperglycemia in diabetic patient (Duncan, 1999) and in alloxan-induced diabetic rats (El-Demerdash et al., 2005). The main contributory factor from garlic that produces these effects is the S-allyl cysteine sulphoxide and sulfur containing amino (Sheela and Augusti, 1992). Interestingly, enough protein peptides with cysteine peptidase activities have also been isolated from *Allium sativum*, which showed specific proteolytic activities on  $\alpha$ -casein and azocasein (Parisi et al., 2008). This opens the possibility of increasing milk protein and digestibility of milk products such as yogurt and cheese, if for instance, *A. sativum* is also

added during and/or after products fermentation. Breakdown of large milk proteins to smaller milk protein molecules is regarded as a positive characteristic of making yogurt because proteolysis causes a progressive release of polypeptides with potential biological activities (Meisel, 1997). However, extensive breakdown of protein by microbial enzymes, either during fermentation or extended refrigeration storage may result in a loss in their biological function (Meisel, 1997). The objectives of the present studies were to compare the effects of the presence of *A. sativum* during camel and cow milk yogurt fermentation on the changes in the physicochemical properties and *in vitro* inhibition of  $\alpha$ -glucosidase and  $\alpha$ -amylase during refrigerated storage.

## 2. Materials and methods

### 2.1. Water extraction of herb

Commercially available dried *A. sativum* powder (10 g) was suspended in 100 ml of distilled water (dH<sub>2</sub>O) and the mixture was incubated overnight in a water bath (70 °C), followed by centrifugation (2000 rpm, 15 min at 4 °C). The clear solution obtained was used as garlic water extract.

### 2.2. Preparation of starter culture

Fresh and pasteurized full cream milk was pre-heated to 41 °C. Yogurt-Mix powder (containing *Lactobacillus acidophilus*, *L. bulgaricus*, *L. casei*, *Bifidobacterium bifidus* and *Streptococcus thermophilus*) was then added into the pre-heated milk. The mixture was incubated for 24 h at 41 °C until pH 4.5 and kept in the refrigerator (4 °C) and used within 7 days.

### 2.3. Preparation of yogurt

Yogurt was prepared by adding 10 ml of garlic water extract into 85 ml of full cream milk containing 5 g of starter culture. The mixture was mixed thoroughly followed by incubation at 41 °C until pH was reduced to 4.5. The same procedures were carried out to prepare plain yogurt (control) by using dH<sub>2</sub>O in place of garlic water extract. Yogurts were then refrigerated (4 °C) for up to 21 days.

### 2.4. Preparation of water yogurt extract

Yogurt sample (10 g) was mixed with 2.5 ml distilled water and the yogurt pH was adjusted to 4.0 using 1 M HCl. The yogurt was then incubated in water bath (45 °C) for 10 min and the precipitated proteins were removed by centrifugation (10,000 rpm, 10 min, 4 °C). The supernatant was harvested and the pH was adjusted to 7.0 using NaOH (0.5 M) followed

by another centrifugation (10,000 rpm, 10 min, 4 °C) to remove residual precipitated proteins and salts. The supernatant was harvested, kept refrigerated and used for subsequent analysis within 24 h.

### 2.5. Measurement of pH and total titratable acid (TTA)

The pH of both plain- and *A. sativum*-yogurt samples was determined using a digital Metler Toledo 320 pH meter. The yogurt samples were mixed with distilled water (1:1 ml) before pH measurement. The TTA (% lactic acid equivalent) was determined by titrating yogurt:dH<sub>2</sub>O (1:9) mixture using 0.1 N NaOH and calculated as follows:

$$\text{TTA}\% = 10 \times V_{\text{NaOH}} \times 0.1 \times 0.009 \times 100\%$$

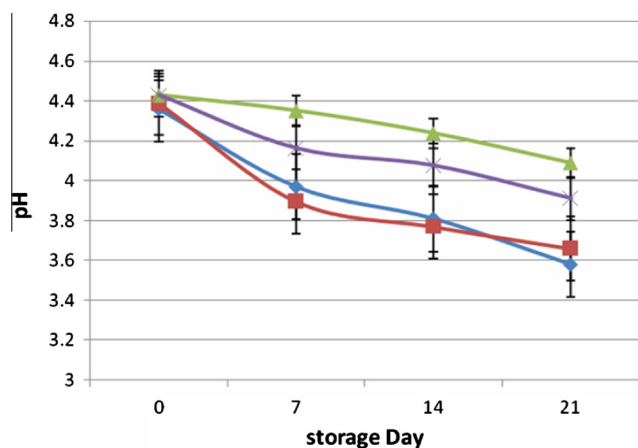
where 10 = dilution factor,  $V_{\text{NaOH}}$  = volume of NaOH required to neutralize the acid and 0.1 = normality of NaOH.

### 2.6. Total phenolic content assay

The total phenolic content was determined as described by Shetty et al. (1995). Briefly, 1 ml of yogurt water extracts was transferred into a test tube and mixed with 1 ml of 95% ethanol and 5 ml of dH<sub>2</sub>O. Folin-Ciocalteu reagent 0.5 ml of 50% (v/v) was added to each sample followed by a thorough mixing. After 5 min, 1 ml of 5% Na<sub>2</sub>CO<sub>3</sub> was added and the reaction mixture was allowed to stand for 60 min. Absorbance at 725 nm was converted to total phenolics expressed in micrograms equivalents of gallic acid per gram (µgGAE/g) sample. Standard curves were established using various concentrations of gallic acid (5–60 µg/ml) in methanol.

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

DPPH inhibition was determined as described by Shetty et al. (1995). Yogurt water extract (250 µl) was added into 3 ml of 60 µM DPPH in ethanol. The mixture was shaken vigorously and allowed to stand at room temperature for several minutes.



**Figure 1** Changes in pH during refrigerated (4 °C) storage. ■ plain-cow milk yogurt (control), ■ *Allium sativum*-cow milk yogurt, ■ plain-camel milk yogurt (control), ■ *Allium sativum*-camel milk yogurt. Values are presented as mean ± SEM ( $n = 3$ ).

The absorbance was then measured at 517 nm. The readings were compared with the controls which contained dH<sub>2</sub>O (250 µl) instead of water yogurt extract. The % inhibition was calculated by

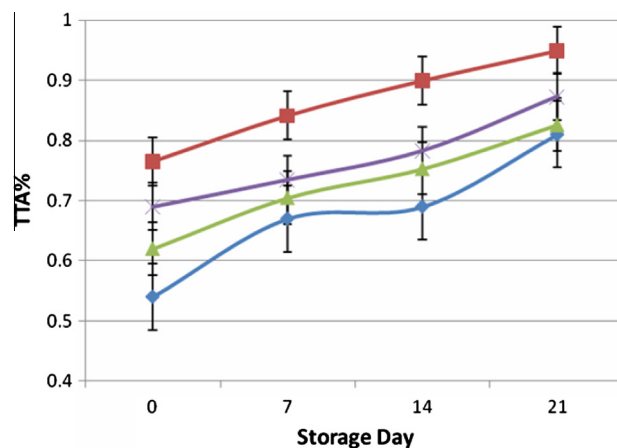
$$\% \text{inhibition} = \frac{[A_{517}^{\text{control}} - A_{517}^{\text{extract}}]}{[A_{517}^{\text{control}}]} \times 100.$$

### 2.8. The *o*-phthaldialdehyde (OPA) assay

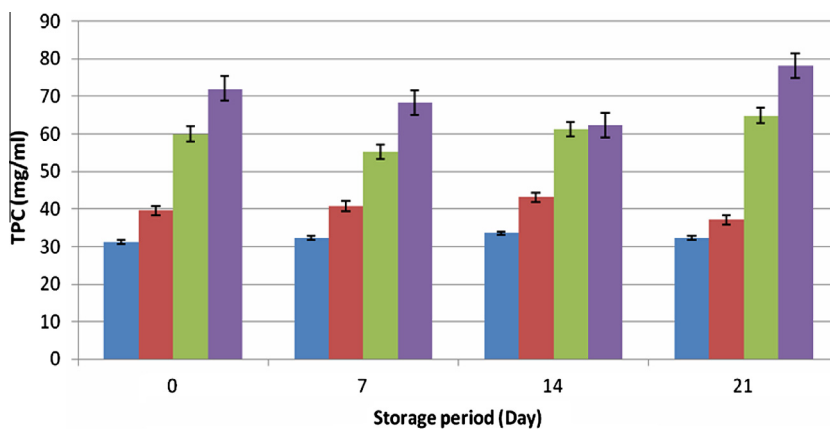
The OPA reagent was prepared as described by Church et al. (1983). The OPA solution was made by combining the following reagents and diluting to a final volume of 50 ml with dH<sub>2</sub>O: 25 ml of 100 mM sodium tetraborate; 2.5 ml of 20% (wt/wt) sodium-dodecyl sulfate (SDS); 40 mg of OPA (dissolved in 1 ml of methanol); and 100 µl of β-mercaptoethanol. This reagent was prepared fresh and used within 2 h of preparation. A small aliquot of water yogurt extract was added directly into 1.0 ml of OPA reagent. The solution was mixed briefly by inversion and incubated for 2 min at room temperature. Absorbance was determined at 340 nm and the peptide concentration was read against tryptone (Difco Laboratories, Sparks, MD, USA) standards (0.125–1.50 mg/ml).

### 2.9. α-Amylase inhibition assay

The α-amylase inhibition assay was measured with starch as carbohydrate substrate in the presence or absence of different modulators (Apostolidis et al., 2006). Water yogurt extracts (500 µl) and 500 µl of 0.02 M sodium phosphate buffer, pH 6.9 with 0.006 M sodium chloride containing 0.5 mg/ml α-amylase solution were pre-incubated at 25 °C for 10 min. Starch solution (1% w/v in 0.02 M sodium phosphate buffer, pH 6.9 with 0.006 M sodium chloride, 500 µl) was then added to each tube followed by incubation at 25 °C for 10 min. The reaction was stopped by mixing with 1.0 ml of dinitrosalicylic acid (DNSA) color reagent. The test tubes were then incubated in a boiling water bath for 7 min followed by the addition of 1.0 ml of 18.2% tartrate solution prior to cooling at room tem-



**Figure 2** Changes in TTA% during refrigerated (4 °C) storage. ■ plain-cow milk yogurt (control), ■ *Allium sativum*-cow milk yogurt, ■ plain-camel milk yogurt (control), ■ *Allium sativum*-camel milk yogurt. Values are presented as mean ± SEM ( $n = 3$ ).



**Figure 3** Changes in total phenolic content ( $\mu\text{g/ml}$ ) in yogurt during refrigerated ( $4^\circ\text{C}$ ) storage. ■ plain-cow milk yogurt (control), ■ *Allium sativum*-cow milk yogurt, ■ plain-camel milk yogurt (control), ■ *Allium sativum*-camel milk yogurt. Values are presented as mean  $\pm$  SEM ( $n = 3$ ).

perature. Distilled water (10 ml) was added to dilute the reaction mixture and the absorbance reading was measured at 540 nm and compared to control which consists of 500  $\mu\text{L}$  of buffer solution in place of the water yogurt extracts. The enzyme inhibition was calculated as follows:

$$\text{Inhibition(\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of extracts}}{\text{Absorbance of control}} \times 100$$

### 2.10. $\alpha$ -Glucosidase inhibition assay

The  $\alpha$ -glucosidase inhibition assay was performed in reference to the method of Apostolidis et al. (2006). Yogurt water extract (500  $\mu\text{l}$ ) and 1000  $\mu\text{l}$  of 0.1 M potassium phosphate buffer (pH 6.90) containing  $\alpha$ -glucosidase solution (1.0 U/ml) were incubated in water bath ( $25^\circ\text{C}$ ) for 10 min. *p*-Nitrophenyl- $\alpha$ -D-glucopyranoside solution (5 mM, 500  $\mu\text{l}$ ) in 0.1 M potassium phosphate buffer (pH 6.90) was then added to each tube and the mixtures were re-incubated at  $25^\circ\text{C}$  for 5 min. Absorbance readings were recorded at 405 nm and the control (buffer in place of sample water extract) was recorded as well. The  $\alpha$ -glucosidase inhibitory activity was expressed as inhibition % as follows:

$$\text{Inhibition\%} = \frac{\text{Absorbance of control} - \text{Absorbance of extracts}}{\text{Absorbance of control}} \times 100$$

### 2.11. Statistical analysis

All experiments were performed at least in triplicates. Data were expressed as mean  $\pm$  standard error. The statistical analysis was performed using one way analysis of variance (ANOVA, SPSS 14.0). The criterion for statistical significance was  $p < 0.05$ .

## 3. Results and discussions

### 3.1. Post-acidification of yogurt

The pH of cow-MY underwent more acidification during refrigerated storage than camel-MY (Fig. 1). The presence of

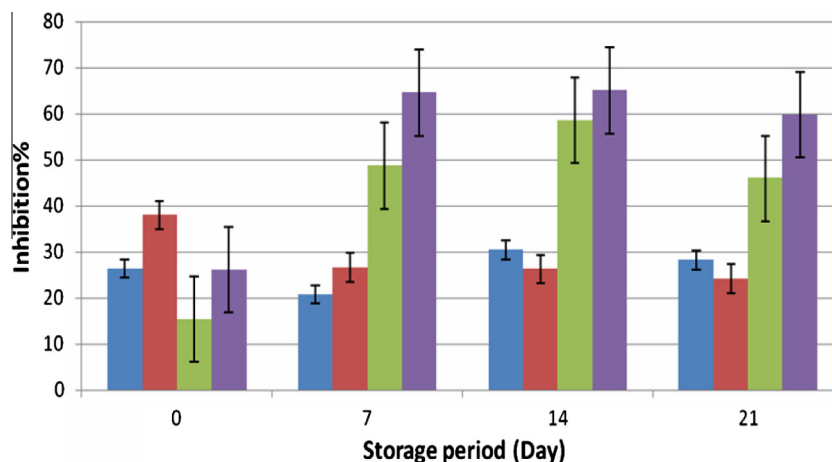
*A. sativum* enhanced ( $p < 0.05$ ) pH reduction in camel-MY but not in cow-MY. In contrast, *A. sativum* enhanced TTA formation in cow milk yogurt ( $p < 0.05$ ), both during fermentation at  $41^\circ\text{C}$  ( $0.8 \pm 0.1$ , day 0) and refrigerated storage (Fig. 2). Post-acidification of the yogurt at  $4^\circ\text{C}$  occurred because lactic acid bacteria (LAB) are active even at this temperature and the residual production of lactic acid, despite small during storage, resulted in noticeable pH decrease and TTA increase (Shah et al., 1995). The relative smaller increase in TTA in camel-MY less than that in cow-MY may further contribute to the fragile coagulum formation in camel-MY compared to yogurts from cow milk. This is in agreement with the previous report Jumah et al. (2001) which showed camel milk viscosity was not changed during gelation process of yogurt.

### 3.2. Total phenolic content assay

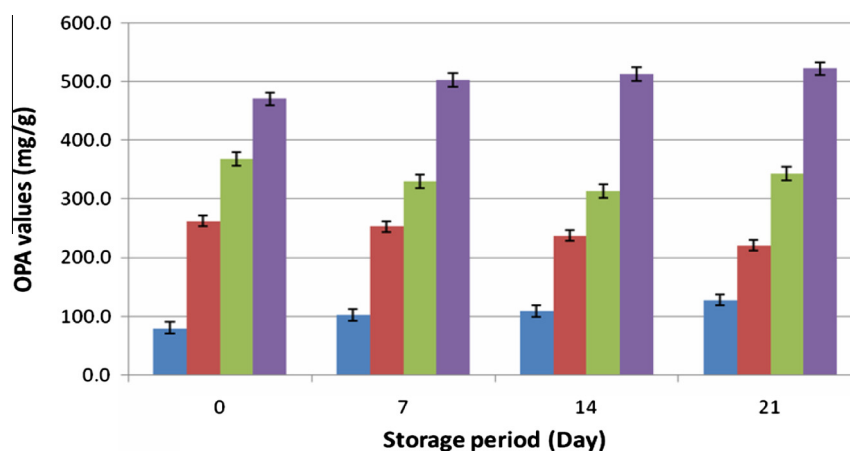
The TPC in camel-MY was about 1.0–1.2-fold higher than that in cow-MY (Fig. 3). Refrigerated storage had minimal effects on TPC in cow-MY. The presence of *A. sativum* elevated ( $p < 0.05$ ) the TPC in both types of yogurts to similar extent. The total phenolic content in milk may be explained by the formation and/or further degradation of polymeric phenolics during fermentation by the yogurt bacteria (Dalling, 1986). In contrast to acidification, the effect of microbe on TPC, both in the absence or presence of *A. sativum*, was only apparent during fermentation but not during storage.

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

The antioxidant activities in fresh camel-MY ( $15.4 \pm 1.3\%$ ) was lower ( $p > 0.05$ ) than cow-MY ( $26.4 \pm 0.7\%$ ; Fig. 4). The presence of *A. sativum* during yogurt formation increased the antioxidant activities in both cow-MY ( $37.9 \pm 0.8\%$ ;  $p < 0.05$ ) and camel-MY ( $26.1 \pm 0.8\%$ ;  $p < 0.05$ ). The antioxidant activities of camel-MY increased to 49–65% whereas cow-MY decreased to 26–28% during 7–21 days of storage. The ability of *A. sativum* extracts to scavenge different radicals



**Figure 4** Changes in antioxidant activity in yogurt (inhibition %) during refrigerated (4 °C) storage. ■ plain-cow milk yogurt (control), ■ *Allium sativum*-cow milk yogurt, ■ plain-camel milk yogurt (control), ■ *Allium sativum*-camel milk yogurt. Values are presented as mean ± SEM ( $n = 3$ ).



**Figure 5** Changes in OPA values (mg/g) in yogurt during refrigerated (4 °C) storage. ■ plain-cow milk yogurt (control), ■ *Allium sativum*-cow milk yogurt, ■ plain-camel milk yogurt (control), ■ *Allium sativum*-camel milk yogurt. Values are presented as mean ± SEM ( $n = 3$ ).

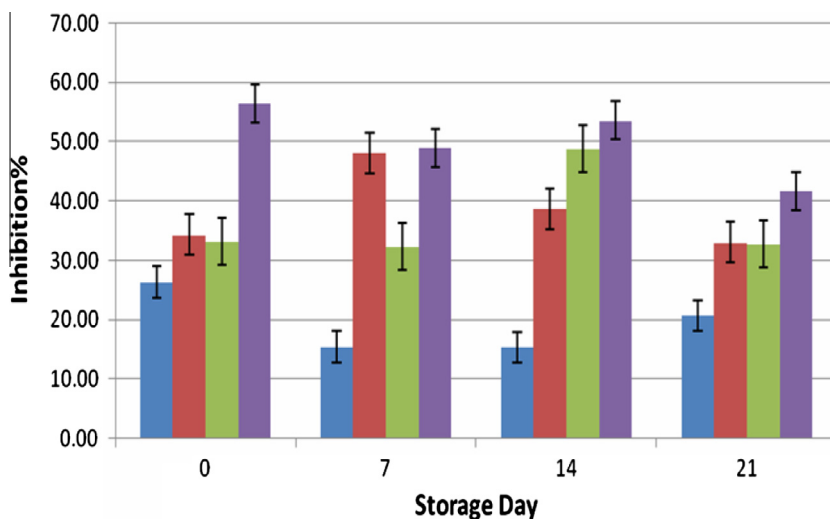
(Bhagyalakshmi et al., 2005; Pedraza-Chaverri et al., 2006) were previously attributed to sulfur, phenolics, flavonoids and terpenoid compounds present in the mature garlic bulbs (Miller et al., 2000; Nuutila et al., 2003; Bozin et al., 2008). Since refrigerated storage tend to result in an increase in higher DPPH inhibition in camel-MY than in cow-MY, it is likely that the presence of *A. sativum* affected the formation of fermented products with antioxidant activities.

#### 3.4. Evaluation of milk protein proteolysis in yogurt by OPA assay

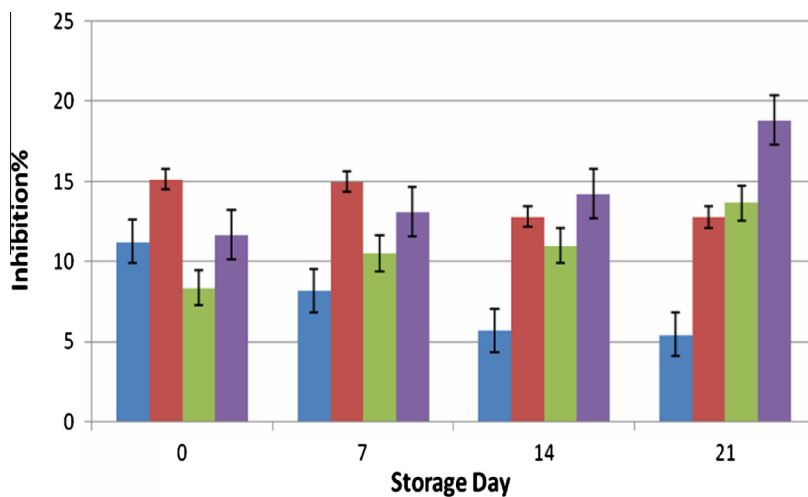
Camel-MY showed 3–4-folds higher OPA values than cow-MY (Fig. 5). Plain yogurt OPA values during refrigerated storage showed a decrease for camel-MY ( $p < 0.05$  for days 7 and 14 of storage) but an increase for cow-MY ( $p < 0.05$  for day 21 of storage). The reverse was true when *A. sativum* was also present in the yogurts in which, in comparison to day 0, the OPA values were higher ( $p < 0.05$ ) for days 7, 14 and 21 for

*A. sativum*-camel-MY and lower ( $p < 0.05$ ) for days 14 and 21 for *A. sativum*-cow-MY. The spectrophotometric absorbance which form the basis of OPA values relate to the released  $\alpha$ -amino groups resulting from the proteolysis of milk proteins. The values obtained have been widely used as a good measurement proteolytic activity of yogurt and probiotic bacteria (Shihata and Shah, 2000). The present studies showed that camel milk protein was more readily broken down during fermentation by yogurt bacteria than cow-milk protein. Camel- and cow-milk proteins are immunologically different (El-Agamy et al., 2009) and this could contribute to the differences in the ease and extent of proteolysis. In 0 day, *A. sativum* increased OPA values of yogurt more for cow-MY (from 80 to 263 mg/g; 3-fold increase) than for camel-MY (from 368 to 471 mg/g; 1.3-fold increase). Enzymes with proteolytic activity in *A. sativum* (Parisi et al., 2008) with different activities for milk proteins in cow and camel milk may partially contribute to the increase in the OPA values. Additionally, extra increase in proteolysis not directly due to *A. sativum* may also occur,





**Figure 6** Changes in  $\alpha$ -amylase inhibitory activity (%) in yogurt during refrigerated (4 °C) storage. ■ plain-cow milk yogurt (control), ■ *Allium sativum*-cow milk yogurt, ■ plain-camel milk yogurt (control), ■ *Allium sativum*-camel milk yogurt. Values are presented as mean  $\pm$  SEM ( $n = 3$ ).



**Figure 7** Changes in  $\alpha$ -glucosidase inhibitory activity (%) in yogurt during refrigerated (4 °C) storage. ■ plain-cow milk yogurt (control), ■ *Allium sativum*-cow milk yogurt, ■ plain-camel milk yogurt (control), ■ *Allium sativum*-camel milk yogurt. Values are presented as mean  $\pm$  SEM ( $n = 3$ ).

possibly via higher release of microbial peptidases by yogurt bacteria in cow's milk than in camel's milk. This needs to be further investigated in future studies.

### 3.5. $\alpha$ -Amylase inhibitory activities in yogurt

In general *A. sativum*-enriched cow- and camel-MYs showed higher  $\alpha$ -amylase inhibition than their respective controls (Fig. 6). The difference in the enzyme inhibition due to *A. sativum* was significant ( $p < 0.05$ ) by days 0, 7, 14 and 21 for cow-MYs, but only by days 0 and 7 for camel-MYs. Refrigerated storage has profound effect on yogurts inhibition of  $\alpha$ -amylase activity. For cow-MY, this was demonstrated by a decrease in  $\alpha$ -amylase inhibition ( $p < 0.05$ ) during storage from 26% in day 0 yogurt to 15% in days 7 and 14 yogurts whereas the presence of *A. sativum* increased

$\alpha$ -amylase inhibition from  $34.3 \pm 3.2\%$  to  $48.1 \pm 2.0\%$  during the first week of storage ( $p < 0.05$ ). Fresh camel-MY was not different from fresh cow-MY but its inhibition of  $\alpha$ -amylase activity did not decline throughout the 21 days storage. *A. sativum* increased  $\alpha$ -amylase inhibition both in fresh ( $p < 0.05$ ) as well as in stored ( $p < 0.05$  for days 7 and 21) camel-MY. S-allyl cysteine sulfoxide (alliin) and a sulfur containing amino acid in garlic were reported to exhibit  $\alpha$ -amylase inhibitory activity (Augusti and Sheela, 1996). This could partially explain the higher  $\alpha$ -amylase inhibitory capacity of cow- and camel-MYs when *A. sativum* was also present compared to their respective plain-MYs. The consumption of fermented foods with  $\alpha$ -amylase inhibitory activity is currently regarded as a practical dietary approach to manage hyperglycemia and diabetes (Djomeni et al., 2006; Fujita et al., 2003). The present study showed that the inclusion of *A. sativum*-

yogurts could increase the therapeutical values of cow- and camel-MYs via the higher and sustained  $\alpha$ -amylase inhibitory activities during storage compared to plain yogurt. Future studies are required to further establish the relative importance of *A. sativum* and products of fermented milk on  $\alpha$ -amylase inhibition. This is because the increase in  $\alpha$ -amylase inhibition by *A. sativum* was not uniform in both cow- and camel-MYs.

### 3.6. $\alpha$ -Glucosidase inhibitory activities in yogurt

Inhibition of  $\alpha$ -glucosidase in cow-MY decreased during storage from  $11.3 \pm 0.4\%$  to  $5.5 \pm 0.2\%$  (Fig. 7).  $\alpha$ -Glucosidase inhibition by *A. sativum*-cow-MY was higher than the control with the small reduction in inhibitory activity (from  $15.2 \pm 0.4\%$  to  $12.8 \pm 0.4\%$ ) during the 21 days storage. In contrast, camel-MY ( $8.4 \pm 0.2\%$ ) had lower  $\alpha$ -glucosidase inhibition than cow-MY ( $p > 0.05$ ) but the inhibitory activity increased ( $p < 0.05$ ) during 21 days of storage to  $13.7 \pm 0.7\%$ . The inhibition of  $\alpha$ -glucosidase by camel-MY was also increased in the presence of *A. sativum* ( $11.7 \pm 1.0\%$ , day 0) with maximum inhibitory activity ( $18.8 \pm 0.5\%$ ) achieved by day 21 of the refrigerated storage. The  $\alpha$ -glucosidase activity in milk (Lee and De Boer, 1994; Bijvoet et al., 1996) is essential to enhance glucose absorption. Fermented milk products however have  $\alpha$ -glucosidase inhibitory activities (Ramchandran and Shah, 2008) and this may explain how fermented milk products help to reduce postprandial hyperglycemia (Kim et al., 2005). The addition of *A. sativum* to cow-MY may be seen to provide two benefits, i.e. to increase the inhibition of  $\alpha$ -glucosidase and to slow down the decrease in this enzyme inhibitory activities during storage. This finding provides further support on the use of plant extracts to increase  $\alpha$ -glucosidase inhibition (Fujita et al., 2003; Djomeni et al., 2006; Shinde et al., 2008). Increasing  $\alpha$ -glucosidase inhibitory activities in camel-MY, both in the absence and the presence of *A. sativum* during storage, were opposite to that seen in cow-MYs. To our knowledge this is the first time such differences were reported and it suggests  $\alpha$ -glucosidase inhibitors were continually produced during storage.

## 4. Conclusion

Cow-MY underwent faster rate of post-acidification than camel-MY during storage. The differences in acidity development and acid content in these two types of yogurt may contribute to the differences in proteolysis and accumulation of fermentation products with anti-oxidants and anti-diabetic enzymes activities during storage.

## Acknowledgment

We acknowledge the financial support of the University of Malaya Research Grant (RG023-090BIO).

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