

# Inclusion of *Allium sativum* in Yogurt and its Effects on Inhibition of Diabetes and Hypertension-associated by Enzymes *in vitro*

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## Abstract

The effects of *Allium sativum* on yogurt formation and subsequent storage (4°C, up to 28 days) on proteolysis, microbial activity, and inhibition of  $\alpha$ -amylase,  $\alpha$ -glucosidase and angiotensin-I converting enzymes were investigated *in vitro*. *Allium sativum* yogurt showed higher rates of pH reduction and increment of titrable acid than control during the incubation at 41°C. Highest proteolysis was observed on day 7 in *Allium sativum* yogurt ( $62.7 \pm 0.80 \text{ mgmL}^{-1}$ ), which was 2-fold higher than the control ( $31.0 \pm 0.96 \text{ mgmL}^{-1}$ ). Bacterial counts in *Allium sativum* yogurt were higher for *Lactobacillus spp.* but lower for *Streptococcus thermophilus* ( $p < 0.05$ ) compared to those in the control throughout refrigerated storage. Highest inhibitory activities for  $\alpha$ -amylase were recorded on day 14 of storage for *Allium sativum* and the control yogurt while highest inhibitory activities for  $\alpha$ -glucosidase were recorded on day 7 of storage for *Allium sativum* and the control yogurt respectively. The highest angiotensin-I converting enzymes activity was observed on day 7 of the refrigerated storage in *Allium sativum* yogurt, and being more potent than the control. *Allium sativum* enhanced the fermentation of yogurt in favor of the population of *Lactobacillus sp.*, stimulated proteolysis of milk proteins, and increased the *in vitro* inhibition of key enzymes associated with diabetes and hypertension.

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## 1.Introduction

Many fermented foods have anti-angiotensin-I converting enzyme (ACE-I) activities, majority of which are attributed to protein breakdown products. Many fermented foods have ACE-I activities, majority of which are attributed to protein breakdown products [1]. As for fermented milks, the health benefits can be attributed to the products of microbial fermentation of milk carbohydrate, fat and protein [2]. Since the metabolic activities of these microbes are instrumental in producing characteristic metabolites, the enhancement of microbial fermentation

of milk can result in the production of more milk products with enhanced health benefits. Inclusion of plants or their extracts in the fermentation can thus increase functional properties of the fermented products as reported for tempeh [3], alcoholic beverages [4] and yogurt [5,6].

Garlic (*Allium sativum* L.) has special place in folk medication, and is used widely in preparing food. The medicinal benefits in frequent uses of garlic may be credited to the anti-microbial [7], anti-thrombotic [8], anti-hypertensive [9], anti-hypergly-

cemic [10], and anti-hyperlipemic [11] activities of this plant. In addition, commercially available garlic preparations in the form of garlic oil, garlic powder, and pills are widely used for certain therapeutic purposes such as for lowering blood pressure, and improving lipid profile [12]. Several of the beneficial effects of garlic on cardiovascular disorders can be attributed to garlic's inhibitory effects on ACE-I [13] as demonstrated *in vitro* [14] and *in vivo*. The health beneficial effects of garlic may also be extended to diabetes and related disorders [15,16]. In fact, complications arising from diabetes mellitus are known to preclude the development of cardio-vascular disorders [8-10]. An increase in peptidyl dipeptidase ACE activity has been reported in diabetic patients and diabetes-induced animals with cardiovascular disorders, nephropathy and retinopathy [8, 10, 11, 17], which lend support to the rational treatment with ACE inhibitors in the prevention of cardiovascular disorders and endothelial vascular dysfunction.

Garlic may influence both the fermentation and the functional properties of fermented milk by virtue of the anti-bacterial effects on microbial growth and also presence of garlic bioactive phytochemicals. Therefore, the present study aimed to ascertain the effects of presence of *A. sativum* on: a) the fermentation of milk by yogurt bacteria, b) the viable microbes in the final product, c) the antioxidant activity, and d) the functional inhibition of key enzymes related to diabetes and hypertension.

## 2. Materials and Methods

### 2.1. Plant material

Commercially available *A. sativum* powder (McCormick) was purchased in dried form from a local market in Malaysia.

### 2.2. Aqueous extraction of *A. sativum*

The powder was homogenized (polytron at maximum setting for 10 seconds) in distilled water in the ratio of 1:10 (wv<sup>-1</sup>). The mixture was incubated overnight (70°C), followed by centrifugation (2000 rpm, 10 min, 4°C), and filtered by Whatman's paper No. 5. The supernatant was harvested, stored at 4°C, and used within 3 days.

### 2.3. Milk and yogurt bacteria

Fresh, pasteurized and homogenized cow milk (4% full fat) was purchased from a local market. The dried yogurt bacteria mixture (Chris Hansen, Denmark) consisted of the following bacteria: *Lactobacillus acidophilus* LA-5, *Bifidobacterium* Bb-12, *Lactobacillus casei* LC-01, and *Streptococcus thermophilus* at the ratio of 4:4:1:1.

### 2.4. Preparation of starter culture

Yogurt starter culture was prepared by inoculating 1 L of pasteurized full fat (4%) milk

with dried yogurt bacteria (1% wv<sup>-1</sup>). The milk-yogurt bacteria mixture was then incubated at 41°C for 12 h and the yogurt formed was stored at 4°C and used as starter culture within 1 week. We routinely found the pH of the starter culture at the range of 4.1-4.3 and viable bacteria to range between 2-5×10<sup>6</sup> cfug<sup>-1</sup> and 6-10×10<sup>8</sup> cfug<sup>-1</sup> for *Lactobacillus spp.* and *S. thermophilus*, respectively, on the 14<sup>th</sup> day of storage.

### 2.5. Preparation of yogurt

The control and herbal yogurt (*A. sativum* yogurt) were both prepared on the same day. Herbal water extract (100 ml) was added into pre-heated (41°C) pasteurized full fat (4%) milk (850 ml) followed by the addition of starter culture (50 ml) containing *S. thermophilus*, *L. acidophilus*, *L. bulgaricus* and *B. bifida*. Full fat milk powder (2 g; 4% fat) was added to modify the milk's solid content to 15% gml<sup>-1</sup>. The extract, starter culture and milk were mixed thoroughly, and aliquoted (100 ml) into disposable plastic containers. Control yogurt was prepared essentially in the same manner as herbal-yogurt with the exception that distilled water (100 ml) was used instead of herbal extract. The prepared yogurts were fermented in water bath (41°C) until pH was reduced to 4.5 followed by refrigeration (4°C) (fresh yogurt or day 0 of storage) up to 28 days.

### 2.6. Preparation of yogurt extract

The control and herbal yogurts (10 g) were homogenized (polytron at maximum setting for 10 seconds) with 2.5 ml of sterile distilled water. The pH of the yogurts was acidified to 4.0 (Mettler-Toledo 320, Shanghai) with HCl (0.1 M). The yogurts were then heated in a water bath (45°C) for 10 min prior to centrifugation (5000 g, 10 min, 4°C). The supernatants were harvested, and NaOH (0.1 M) was added to adjust the pH to 7.0. The yogurt water extracts were re-centrifuged (5000 g, 10 min, 4°C), and the supernatants were harvested and stored at -20°C for required analysis.

### 2.7. pH and titrable acid (TA) determination

The yogurt samples were initially homogenized (polytron at maximum setting for 10 seconds) in distilled water 1:1 and 1:9 ratio prior to pH or TA determination, respectively [18]. The amount of acid produced was calculated as follows:

Lactic acid (%) =  $V_{\text{NaOH}} \times 0.1 \text{ N} \times 10 \times 0.009 \times 100$   
Where, V is the volume of NaOH required to neutralize the acid.

### 2.8. Quantification of total phenolic content

Total phenolic content (TPC) in the yogurt samples was quantified as described by Shetty, Vatter, & Clydesdale [11]. The reaction mixture

was prepared by mixing 1 ml of the yogurt extract with 1 ml of 95% ethanol and 5 ml of distilled water (dH<sub>2</sub>O) in a test tube. Folin-Ciocalteu reagent (diluted 1:1 with distilled water) was added to each sample followed by thorough mixing using a vortex. After 5 minutes, 1 ml of 5% Na<sub>2</sub>CO<sub>3</sub> was added to the reaction mixture; then these mixtures were left to stand for 60 min at room temperature. The absorbance readings were done at 725 nm. Standard curves were prepared by various concentrations of gallic acid (5-60 µgml<sup>-1</sup>) in methanol. The results were reported as microgram equivalents of gallic acid (GAE) per millilitre of the sample.

## 2.9. Antioxidant activity of yogurt extract by 1,1-diphenyl-2-picrylhydrazyl radical inhibition (DRI) assay

The antiradical activity of yogurt extracts was determined using 1,1-diphenyl-2-picrylhydrazyl (DPPH) method [17]. The yogurt extracts (250 µl) were added into 3 ml of 60 mmol<sup>-1</sup>L DPPH in ethanol (Sigma Aldrich, Germany). The decrease in absorbance was monitored at 517 nm until a constant reading was obtained. The constant readings for the yogurt extracts and control (consisting of 250 µl of water instead of extract) were used in the calculation of the inhibition percentage of DPPH (%DPPH) oxidation or free radical scavenging capacity (RSC) using the following formula:

$$\text{RSC (\%)} = 100 \times (A_{\text{blank}} - A_{\text{sample}} / A_{\text{blank}})$$

## 2.10. O-Phthalaldehyde (OPA) assay

Proteolysis products present in the yogurt water extracts were measured using o-phthalaldehyde (OPA), which reacts with primary amines (functional group of peptides) in the presence of β-mercaptoethanol [18]. Sodium tetraborate (Borax; 25 ml 100 mM), sodium dodecyl sulphate (2.5 ml 20% of ww<sup>-1</sup>), 1.1 ml OPA (40 mg OPA dissolved in 1 ml methanol) and 100 µl β-mercaptoethanol (Sigma-Aldrich, Germany) were mixed in 21.4 ml dH<sub>2</sub>O [20]. This reagent, prepared fresh on the day of assay, was protected from direct light, and used within 2 h of preparation. Aliquots of the yogurt water extract (30 ml containing 5-100 mg proteins) were added directly to OPA reagent (1.0 ml) in a 1.5 ml quartz cuvette, and the solutions were mixed briefly by inversion prior to 2 min incubation at room temperature followed by absorbance (340 nm) measurement. The peptide concentration was estimated against the tryptone (Difco Laboratories, Sparks, MD, USA) standards (0.125-1.50 mgml<sup>-1</sup>).

## 2.11. Enzyme inhibition

### 2.11.1. Determination of the angiotensin I-converting enzyme's (ACE-I) inhibition activity

Rabbit lung was used as a source of ACE-I. Fresh rabbit lung, removed immediately from the rabbit post-mortem, was snap-frozen to -20°C

followed by grinding using pestle and mortar, and subsequently, homogenized (polytron at maximum setting for 10 seconds) with ice-cold 0.05 M potassium phosphate buffer, pH 8.3 [21]. The homogenized rabbit lung was then centrifuged for 60 min at 20000 g. The clear red supernatant possessed high ACE activity, and was aliquoted into 1ml ampoules prior to freezing (-20°C) for further analysis. All enzyme assays were carried out using yogurt water extracts at three different dosages. The ability of the yogurt extract to inhibit the activity of ACE-I to hydrolyse the substrate furanocryloyl tripeptide Phe-Gly-Gly (FAPGG; Sigma-Aldrich, St Louis, MO, USA) in the absence (dH<sub>2</sub>O) or presence of yogurt water extract (300, 250 or 125µL) was measured according to the spectrophotometric (Thermo-spectronic 10 UV, 190-1100 nm, USA) method [22]. Appropriate buffer volumes (50 or 175 µl, respectively) were added to the last two dosages to make up the volume to 300 µl. Absorbance at 340nm was recorded every 5 min, and the linear rate of absorbance change was used in the calculation of enzyme activity (unit absmin<sup>-1</sup>). ACE-I inhibition was calculated as follows:

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

Where,

A = Abs<sub>340nm</sub> (ACE solution in the buffer),

B = Abs<sub>340nm</sub> (buffer),

C = Abs<sub>340nm</sub> (ACE solution with the ACE inhibitory component added in the buffer), and

D = Abs<sub>340nm</sub> (ACE inhibitory component in the buffer)

### 2.11.2. α-amylase and α-glucosidase inhibition assays

The α-amylase and α-glucosidase inhibition assays were adapted from Apostolidis, Kwon & Shetty [17] with absorbance measured at 540 nm and 404 nm, respectively. Three different doses of yogurt water extract used were 500, 250 and 125µl. appropriate buffer volumes (250 or 375µL, respectively) were added to the last two dosages to make up the volume to 500µl. The inhibition (%) of enzymes was calculated as below:

$$\text{Inhibition (\%)} =$$

$$(\text{Absorbance control} - \text{Absorbance of extracts} / \text{Absorbance of control}) \times 100$$

### 2.11.3. Determination of IC<sub>50</sub>

Enzyme inhibition, expressed in terms of IC<sub>50</sub>, is defined as the protein concentration (µgml<sup>-1</sup>) in the sample required to inhibit 50% of the enzyme activity. The protein concentration in all yogurt extracts was determined according to Bradford [22]. In the present study, each sample was adjusted to at least three levels of concentrations by standard volume dilution. The protein content of each sample was determined (µgml<sup>-1</sup>), and the IC<sub>50</sub> was then

subjected to the non-linear adjustment programme (PRISM, version 4.02) for Windows (GraphPad Software, Inc. San Diego, CA, USA)) to determine the protein concentration in the sample ( $\mu\text{gml}^{-1}$ ) required inhibiting 50% of the ACE activity.

## 2.12. Microbial viable cell count in yogurts

Bacterial enumeration was determined by the spread-plating diluted ( $10^1$  to  $10^8$  in sterile peptone water (2%  $\text{wv}^{-1}$ ) as diluent) samples of yogurts on agar. M.R.S. agar (anaerobic, 72 h, 37°C, Oxoid, CM0361, Basingstoke, England) was used to enumerate *Lactobacillus* spp. whereas M17 agar (aerobic, 24 h, 37°C, Oxoid, CM0785, Basingstoke, England) was used to enumerate *S. thermophilus*. Twenty grams of buffered peptone water was mixed with 1 L of distilled water, and the mixture was distributed into the final tubes followed by autoclaving (121°C for 20 min).

## 2.13. Statistical analysis

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

## 3. Results and Discussion

### 3.1 Effect of *A. sativum* on change of acidification of yogurt during the refrigerated storage

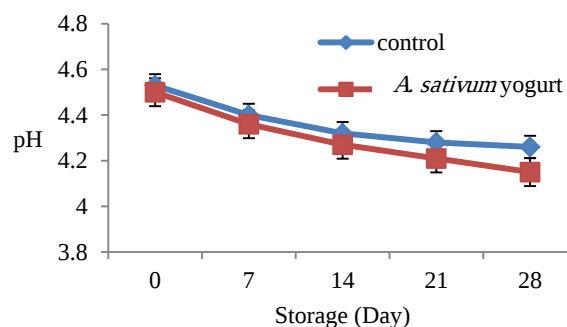
PH value and acidity of the yogurt containing *A. sativum* and the control were measured during 4 weeks of storage, and the results are shown in Fig 1. Initial pH value for *A. sativum* yogurt and control was  $4.53 \pm 0.01$  and  $4.53 \pm 0.02$ , respectively. Yogurts TA value was in the range of  $0.88 \pm 0.03$  and  $0.92 \pm 0.01$  lactic acid equivalent on day 0 of storage (fresh yogurt; at the point of incubation termination) (Fig. 2). Refrigerated storage for 28 days resulted in continued decrease in pH, and increase in the lactic acid production of both yogurts. *A. sativum* yogurt had the faster rate of pH reduction followed by the control. This resulted in the pH of *A. sativum* yogurt being the lower ( $4.15 \pm 0.01$ ) on day 28 of storage followed by the control ( $4.26 \pm 0.01$ ). The rate of acid formation in the *A. sativum* yogurt was higher than in the control.

The high rate of change in pH with storage days could be due to the decomposition of fermentable substrates and sugars by microorganisms, especially *Lactobacillus* species, which ferment carbohydrates to produce energy and principally lactic acid. Higher increase ( $p \geq 0.05$ ) of TA formation was observed in the *A. sativum* yogurt than in the control

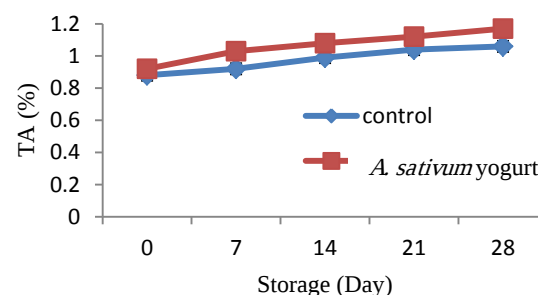
( $1.17 \pm 0.1\%$  and  $1.06 \pm 0.02\%$  for *A. sativum* yogurt and control, respectively) in day 28 of storage. Lactic acid bacteria were reported as the major microorganisms that lead to milk fermentation and play important role to determine the quality of yogurt. Garlic is used most frequently as an ingredient in yogurt. Since ground garlic is used in yogurt, alliin is changed to allicin. Allicin takes part in desirable fermentation of yogurt as it inhibits the growth of aerobic microorganisms derived from the sub-ingredients, and thus helps to propagation of LAB in milk fermentation. LAB was reported to be least sensitive to the inhibitory activity of garlic than other microorganisms [23]. However, different LABs showed different sensitivities to garlic. *A. Sativum* can either delay or stimulate milk fermentation depending on the LAB that is accounted. LAB produces organic acid,  $\text{CO}_2$  and ethanol, in addition to lactic acid, from hexoses or other sugars [24].

### 3.2. Effect of *A. sativum* on total phenolic content & antioxidant capacity of yogurt during storage

The total phenolic content in the homogenized yogurt extracts was analyzed by Folin-Ciocalteu method. The results indicated that the *A. sativum* yogurt had higher, but not significant, phenolic content when compared to the control.



**Figure 1.** Changes of pH in the plain yogurt (control) and *A. sativum* yogurts during 28 days of storage at 4°C. Each experiment was repeated three times and values are means  $\pm$  SD



**Figure 2.** Changes of titrable acid (TA) in the plain yogurt (control) and *A. sativum* yogurts during 28 days of storage at 4°C. Each experiment was repeated three times and values are means  $\pm$  SD

Previous studies had same reports [17]. Refrigerated storage increased TPC in both yogurts on day 14 of storage ( $15.01 \pm 1.20$  and  $12.43 \pm 0.84$ , respectively), followed by gradual decrease during the next 7 days and increase again in day 28 of storage. Since plain-yogurt contains no plant extracts, the TPC values in plain-yogurt reflect the phenolic compounds related to milk protein breakdown. Tyrosine, for instance, has a phenolic side chain suggested to give rise to the reading in TPC [6].

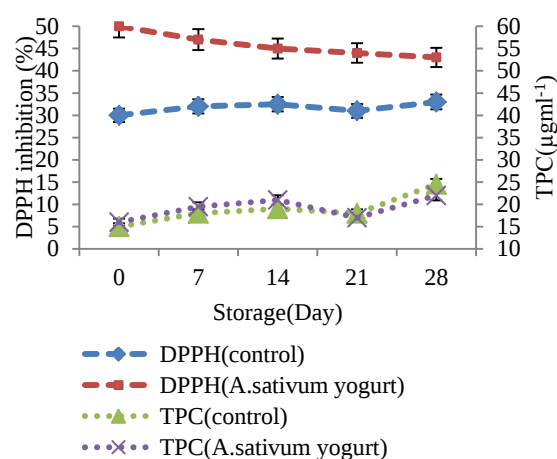
*A. sativum* yogurt showed higher inhibition ( $p < 0.05$ ) of DPPH oxidation *in vitro* (45-52%) than that shown by the control (31-35%) during the refrigerated storage. *A. sativum* is known to be rich in phenolic compounds [25]; however, in the present study, the amount added into the yogurt was not large enough to cause differences in TPC in the control and *A. sativum* yogurts, which was in the range of  $8\text{--}18 \mu\text{gml}^{-1}$  during the refrigerated storage (Fig. 3). Since the organosulfur compounds (diallylsulfide and s-allylcysteine) of *A. sativum* were quantified by the Folin-method, most of the increase in the antioxidant activities of *A. sativum* yogurt can be attributed to these garlic compounds [26].

The presence of *A. sativum* during yogurt formation increased the antioxidant activities in *A. sativum* yogurt compared to the control ( $53.2 \pm 1.2$  and  $31.1 \pm 1.5$  %, respectively; Fig. 3).

Refrigerated storage resulted in gradual decreased antioxidant capacity ( $p < 0.05$ ) with prolonged storage and maximum values capacity recorded by *A. sativum* yogurt in contrast to the control, which was relatively unchanged throughout the storage period. The ability of *A. sativum* extracts to scavenge different radicals [27,28] was previously attributed to sulfur, phenolics, flavonoids and terpenoid compounds present in the mature garlic bulbs [29-31]. On the other hand, the free radical scavenging activity in yogurt can be attributed to the fermentation products as demonstrated by the two-fold increase in antioxidant activity in the plain yogurt (control) compared to the fresh raw milk ( $8.11 \pm 0.23$  %, table not shown).

### 3.3. Effect of *A. sativum* on viable cell count in yogurt during the storage

The changes in the viable cell counts (VCC) of *Lactobacillus* spp. and *S. thermophilus* are presented in Table 1. The numbers of *Lactobacillus* spp. and *S. thermophilus* in fresh *A. sativum* yogurt ( $8.2 \pm 0.4 \times 10^8$  and  $8.9 \pm 0.5 \times 10^6$  cfu-1ml, respectively) were marginally higher ( $p \geq 0.05$ ) than in the control ( $7.8 \pm 0.5 \times 10^8$  and  $8.85 \pm 0.3 \times 10^6$  cfu $\text{ml}^{-1}$ , respectively). The survival of *Lactobacillus* spp. decreased gradually to  $6.9 \pm 0.2 \times 10^8$  and  $4.9 \pm 0.3 \times 10^8$  cfu $\text{ml}^{-1}$  in *A. sativum* yogurt and control, respectively, by day 28 of storage.



**Figure 3.** Total phenolic content (TPC) and antioxidant capacity (% inhibition of DPPH oxidation) by the plain yogurt (control) and *A. sativum* yogurts during 28 days of refrigerated (4°C) storage. Each experiment was repeated three times, and values are means  $\pm$  SD.

VCCs of *S. thermophilus* in fresh yogurts were marginally higher in the *A. sativum* yogurt than in the control ( $8.9 \pm 0.5 \times 10^6$  and  $8.4 \pm 0.6 \times 10^6$  cfu $\text{ml}^{-1}$ , respectively). Both yogurts had increased viable *S. thermophilus* count on day 7 of storage in comparison to those in the fresh yogurt. Extended storage resulted in gradual decrease in *S. thermophilus* count in both yogurts until day 28 of storage.

Refrigerated storage resulted victims in the cell numbers for both *Lactobacillus* spp. and *S. thermophilus*, and thus made the yogurt to have limited shelf life. Plant ingredients such as guar gum and cocoa or compound from plant (dextrose) were found to enhance the viability of probiotic in dairy products. In the present study, the inclusion of *A. sativum* in the yogurt increased LAB counts compared to the control. However, the reduction of *Lactobacillus* spp. VCC during the storage for both types of yogurt could be associated with the post-acidification, which causes further reduction in pH. The reduction in *S. thermophilus* by days 14, 21 and 28 of storage for *A. sativum* yogurt, respectively, may be attributed to the accumulation of organic acids and waste products such as hydrogen peroxide produced by the bacterial activity. Anti-microbial activities by most herbs and spices are attributed to the phenolic compounds present in these plants. These phenolic compounds could either alter microbial cell permeability, thus compromising the integrity of cells or interfering with the membrane functions important in the cell functions such as electron transport, nutrient uptake, protein and nucleic acid synthesis, and enzyme activity [32].

**Table 1.** Comparison of the effects of *A. sativum* on the viability of *Lactobacillus* spp. (108cfu ml<sup>-1</sup>) and *Streptococcus thermophilus* (106cfu ml<sup>-1</sup>) in *A. sativum* yogurt and control during the storage at 4°C.

| Storage (day)           | <i>Lactobacillus</i> sp. counts (log <sub>10</sub> CFUml <sup>-1</sup> ) |                        |                       |                        |                       | <i>S. thermophilus</i> counts (log <sub>10</sub> CFUml <sup>-1</sup> ) |                       |                       |                       |                       |
|-------------------------|--------------------------------------------------------------------------|------------------------|-----------------------|------------------------|-----------------------|------------------------------------------------------------------------|-----------------------|-----------------------|-----------------------|-----------------------|
|                         | 0                                                                        | 7                      | 14                    | 21                     | 28                    | 0                                                                      | 7                     | 14                    | 21                    | 28                    |
| Control                 | 7.8±0.5 <sup>ac</sup>                                                    | 7.7±0.4 <sup>ac</sup>  | 6.3±0.4 <sup>bc</sup> | 5.03±0.4 <sup>bc</sup> | 4.9±0.3 <sup>ac</sup> | 8.4±0.6 <sup>ac</sup>                                                  | 8.9±0.3 <sup>ac</sup> | 7.4±0.2 <sup>bc</sup> | 6.9±0.1 <sup>ac</sup> | 6.2±0.3 <sup>ac</sup> |
| <i>A.sativum</i> yogurt | 8.2±0.4 <sup>ac</sup>                                                    | 9.11±0.2 <sup>bd</sup> | 7.7±0.8 <sup>ad</sup> | 7.2±0.2 <sup>ad</sup>  | 6.9±0.2 <sup>ad</sup> | 8.9±0.5 <sup>ac</sup>                                                  | 9.7±0.4 <sup>bd</sup> | 8.6±0.4 <sup>ad</sup> | 7.9±0.4 <sup>bd</sup> | 7.2±0.3 <sup>ad</sup> |

<sup>a b</sup> Means in the same row with different alphabets are significantly different ( $p < 0.05$ ) for each type of yogurt.

<sup>c d</sup> Means in the same column with different alphabets are significantly different ( $p < 0.05$ ) for the same storage day.

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

*A. sativum* is rich in phenolic compounds, and is known to have anti-microbial actions against several pathogenic bacteria [33] but apparently not on the yogurt bacteria and probiotic as shown in the present study.

### 3.4. Proteolysis of milk protein in yogurt

The OPA-based spectrophotometric assay, which quantitates  $\alpha$ -amino groups, reflects the extent of proteolytic activity [20]. OPA values were higher in fresh yogurts in the presence of *A. sativum* (54.8±2.48mgml<sup>-1</sup>) compared to the control (27.0±1.55 mgmL<sup>-1</sup>,  $p < 0.05$ ). OPA values were highest on day 7 of storage, followed by a consistent reduction until day 28 of storage for both yogurts (Table 2). This suggests that *A. sativum* enhanced microbial metabolic activity not only during the fermentation but also during the refrigerated storage. Since, *Lactobacillus* spp. and *S. thermophilus* counts were not significantly different between the two yogurt treatments, higher OPA value in *A. sativum* yogurt may implicate an increase in the metabolic activities of these bacteria in the presence of *A. sativum*. The high OPA values coincide with the lowest pH and highest percentage of TA on day 7 (Figs.1 and 2). Reduced OPA values in both treatments from day 7 to day 14 may be a combination of reduced release of exogenous proteases as a consequence of increased acidity that restricts further growth of the microbes and continued uptake of peptides by microbes for nitrogenous sources [34]. In addition, accumulation

of waste products such as hydrogen peroxide [35] may also restrict the growth and survival of yogurt bacteria; this was also evident in the present study.

### 3.5. Angiotensin-Converting Enzyme Inhibition (ACE-I)

ACE-I activity was inhibited by yogurts in a dose-dependent manner (data not shown).

*A. sativum* extracts (at the same concentration used in *A. sativum*-yogurt) exert small inhibition on ACE activity (13%), and this could be attributed to phytochemicals such as mycerene and allicin in *A. sativum* [36]. *A. sativum* yogurt showed much higher ACE-I inhibition (62%) compared to the control (46%).

Refrigerated storage increased the inhibitory effects of both yogurts on *in vitro* enzyme activity (Table 2). The IC<sub>50</sub> in fresh control (day 0; 15.4± 0.7 mgg<sup>-1</sup>) was reduced ( $p < 0.05$ ) to 9.7±0.1 mgg<sup>-1</sup> on day 7 of storage but increased to 12.7±0.6 mgg<sup>-1</sup>, 14.7±0.7 mgg<sup>-1</sup> and 27.7±1.3 mgg<sup>-1</sup> on days 14, 21 and 28, respectively. Lowest IC<sub>50</sub> value coincides with highest OPA values observed on day 7.

*A. sativum* yogurt showed similar changes in the potency of ACE inhibition as a result of refrigerated storage. Lowest IC<sub>50</sub> value (6.9±0.23 mgg<sup>-1</sup>) was observed on day 7 of storage, which was lower ( $p < 0.05$ ) than that of the control. Fresh yogurt is known to contain biologically active proteins, which increase during the storage as a result of microbial proteolysis of the milk proteins [37].

**Table 2.** Comparison of the effects of *A. sativum* on OPA peptides and angiotensin-1 converting enzyme (ACE) in *A. sativum* yogurt and control during storage at 4°C

| Storage (day)           | OPA peptides (mgml <sup>-1</sup> ) |                        |                        |                        |                        | IC <sub>50</sub> (mgg <sup>-1</sup> ) for ACE <sup>2</sup> |                       |                        |                        |                        |
|-------------------------|------------------------------------|------------------------|------------------------|------------------------|------------------------|------------------------------------------------------------|-----------------------|------------------------|------------------------|------------------------|
|                         | 0                                  | 7                      | 14                     | 21                     | 28                     | 0                                                          | 7                     | 14                     | 21                     | 28                     |
| Control                 | 27.0±1.6 <sup>ac</sup>             | 31.0±1.0 <sup>bc</sup> | 25.8±0.7 <sup>ac</sup> | 26.8±0.8 <sup>ac</sup> | 25.7±0.9 <sup>ac</sup> | 15.4±0.7 <sup>bc</sup>                                     | 9.7±0.1 <sup>ac</sup> | 12.7±0.6 <sup>ac</sup> | 14.7±0.7 <sup>ac</sup> | 27.7±1.3 <sup>bc</sup> |
| <i>A.sativum</i> yogurt | 54.8±2.5 <sup>ad</sup>             | 62.7±0.8 <sup>bd</sup> | 50.3±1.0 <sup>ad</sup> | 53.0±1.9 <sup>ad</sup> | 50.5±1.8 <sup>ad</sup> | 12.9±0.4 <sup>bc</sup>                                     | 6.9±0.2 <sup>ac</sup> | 10.1±0.9 <sup>bc</sup> | 12.1±0.4 <sup>bc</sup> | 17.0±0.3 <sup>ad</sup> |

<sup>a b</sup> Means in the same row with different alphabets are significantly different ( $p < 0.05$ ) for each type of yogurt.

<sup>c d</sup> Means in the same column with different alphabets are significantly different ( $p < 0.05$ ) for the same storage day.

<sup>1</sup> O-phthalaldehyde assay for peptides using calibrated curve of tryptone standards

<sup>2</sup> IC<sub>50</sub> values were calculated by subjecting ACE-inhibition by 125, 250 and 500ul yogurt water extracts to a non-linear adjustment programme (PRISM version 4.02 for Windows (GraphPad Software, Inc. San Diego, CA, USA).

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

The greater inhibitory effects of *A. sativum* yogurt on ACE-I activity suggest positive effects of *A. sativum* on yogurt bacteria during the fermentation and refrigerated storage, as evident from enhanced acidification of yogurt and proteolysis of milk protein.

The difference in IC<sub>50</sub> values for ACE-I in fresh yogurts (15.4±0.66 and 12.9±0.37 mgg<sup>-1</sup> for the control and *A. sativum* yogurts, respectively) indicates that peptides in *A. sativum* yogurt were more active than those in the control yogurt.

Furthermore, the higher proteolytic activity in the *A. sativum*-yogurt compared to the control during the first 7 days of refrigerated storage could have resulted in the formation of more bioactive peptides in the treatment sample (*A. sativum* yogurt) (IC<sub>50</sub> values of 9.7±0.12 mgg<sup>-1</sup> and 6.9±0.23 mgg<sup>-1</sup> for the control and *A. sativum* yogurts, respectively). Fermented milk products, containing biologically active peptides valyl-prolyl-proline (Val-Pro-Pro) and isoleucyl-prolyl-proline (Ile-Pro-Pro), were shown to lower blood pressure in spontaneously hypertensive human model. These peptides act as ACE inhibitors, which will bind to the enzyme competitively, and in the case of ACE-I assay, prevent the breakdown of substrate, furanacryloyl-Phe-Gly-Gly (FAPGG) to furanacryloyl-Phe (FAP) and Gly-Gly. Hence, variations in the proteolytic capabilities of different yogurts, such as those demonstrated in the present study, may be related to the corresponding variations in the ACE-I inhibitory potential of yogurt extracts reported earlier [38]. The length of storage time and conditions of the storage were found to have crucial effect on the formation and further breakdown of protein into inactive peptides and amino acids [39]. Both the *A. sativum* and control yogurts showed inhibitory activities, which may be related to the formation of new small protein fragments from the proteolysis of milk proteins during the storage at 4°C. In fact, there was correlation between the ACE inhibition of yogurts (IC<sub>50</sub>) and the OPA values that were found to be present for both of the *A. sativum* and control yogurts (R<sup>2</sup>=0.48 and 0.57, respectively). In the case of *A. sativum* yogurt, faster rate of pH reduction and

higher TA contents (Figs. 1 and 2) suggested faster microbial growth and possibly higher microbial population at the end of the fermentation. Detailed studies are required to establish the changes in yogurt microbial population during the fermentation and refrigerated storage to substantiate the relationship between the formation of bioactive peptides and ACE-I activity. Nevertheless, the present study showed that the proteolysis of milk proteins has occurred in the presence of *A. sativum* may in a manner such that the quantity (Table 2; OPA peptides) and quality (Table 2; IC<sub>50</sub>) of peptides formed were advantageous with respect to the inhibition of ACE-I activity.

### 3.6. $\alpha$ -Amylase and $\alpha$ -glucosidase inhibition

The IC<sub>50</sub> values were reduced during the refrigerated storage of yogurts for  $\alpha$ -amylase (the first 14 days) and  $\alpha$ -glucosidase (the first 7 days) followed by an increase in the fresh yogurts thereafter (Table 3). The IC<sub>50</sub> value of *A. sativum* yogurt and control for  $\alpha$ -amylase on the day 14 of storage was 13.7±1.99 and 26.3±2.15 mgg<sup>-1</sup>, respectively, whereas this value for  $\alpha$ -glucosidase on day 7 was 120.7±22.71 mgg<sup>-1</sup> and 192.3±33.24 mgg<sup>-1</sup>, for *A. sativum* yogurt and control respectively. The inhibition of both  $\alpha$ -amylase and  $\alpha$ -glucosidase activities by *A. sativum* yogurt was higher ( $p < 0.05$ ) than that of the control yogurt throughout the 28 days of storage. *A. sativum* by itself showed little inhibitory effects ( $p \geq 0.05$ ) on the inhibition of  $\alpha$ -amylase and  $\alpha$ -glucosidase (data not shown).

This suggests that the phytochemical compounds in *A. sativum* alone had little effect on the inhibition of the digestive enzymes, and that the observed enzyme inhibiting activity by *A. sativum* yogurt may be attributed to fermentation products. The inhibition of  $\alpha$ -glucosidase, but not  $\alpha$ -amylase, by *A. sativum* yogurt can be attributed to the peptide contents in this yogurt since both enzymes were found to be correlated ( $r^2 = 0.45$ ).

**Table 3.** Comparison of the effects of *A. sativum* on IC<sub>50</sub> of  $\alpha$ -amylase and  $\alpha$ -glucosidase in *A. sativum* yogurt and control during storage at 4°C

| Storage (day)            | IC <sub>50</sub> (mgg <sup>-1</sup> ) for $\alpha$ -amylase <sup>2</sup> |                        |                        |                        |                        | IC <sub>50</sub> (mgg <sup>-1</sup> ) for $\alpha$ -glucosidase <sup>3</sup> |                          |                          |                         |                          |
|--------------------------|--------------------------------------------------------------------------|------------------------|------------------------|------------------------|------------------------|------------------------------------------------------------------------------|--------------------------|--------------------------|-------------------------|--------------------------|
|                          | 0                                                                        | 7                      | 14                     | 21                     | 28                     | 0                                                                            | 7                        | 14                       | 21                      | 28                       |
| Control                  | 37.5±2.5 <sup>bc</sup>                                                   | 27.9±1.9 <sup>ac</sup> | 26.3±2.2 <sup>ac</sup> | 44.2±1.7 <sup>bc</sup> | 60.2±3.1 <sup>ac</sup> | 480.0±2.5 <sup>bc</sup>                                                      | 192.3±33.2 <sup>ac</sup> | 290.7±15.6 <sup>bc</sup> | 300.7±2.0 <sup>bc</sup> | 364.3±40.3 <sup>ac</sup> |
| <i>A. sativum</i> yogurt | 28.0±2.2 <sup>bc</sup>                                                   | 18.6±2.1 <sup>d</sup>  | 13.7±2.0 <sup>ad</sup> | 31.5±2.5 <sup>bd</sup> | 45.5±2.8 <sup>ad</sup> | 302.7±63.8 <sup>bd</sup>                                                     | 120.7±22.7 <sup>ad</sup> | 143.7±7.1 <sup>ad</sup>  | 148.7±5.2 <sup>ad</sup> | 200.0±27.6 <sup>ad</sup> |

<sup>a,b</sup> Means in the same row with different alphabets are significantly different ( $p < 0.05$ ) for each type of yogurt.

<sup>c,d</sup> Means in the same column with different alphabets are significantly different ( $p < 0.05$ ) for the same storage day.

<sup>1</sup> IC<sub>50</sub> values were calculated by subjecting ACE-inhibitions caused by 125, 250 and 500µl yogurt water extracts to a non-linear adjustment programme (PRISM version 4.02 for Windows (GraphPad Software, Inc. San Diego, CA, USA)).

<sup>2</sup> Porcine pancreatic  $\alpha$ -amylase (Sigma-Aldrich, USA)

<sup>3</sup> Baker yeast  $\alpha$ -glucosidase (Sigma-Aldrich, USA)

Each experiment was repeated three times and values are means ± SD

Furthermore, the correlation of these two enzymes' inhibition with the antioxidant activities of yogurts is almost nonexistence ( $r^2 < 0.10$ ), although there are reports showing that antioxidants in dietary products may contribute to certain level of diabetic inhibitory potential [40].

#### 4. Conclusion

The presence of *A. sativum* enhanced fermentation and post-acidification of yogurt during the refrigerated storage via the retention of the viability of yogurt bacteria. *A. sativum* increased the capacity of yogurts to inhibit ACE-I, amylase and glucosidase *in vitro*; and these are correlated with the extent of milk protein proteolysis during fermentation and refrigerated storage. Inhibition of ACE-I is considered a useful therapeutic approach in the treatment of high blood pressure in both diabetic and non-diabetic patients. In addition, production of foods with ACE-inhibitory peptides such as yogurt may also be beneficial for individuals with increased blood pressure. ACE-inhibitory peptides are encrypted within the intact milk proteins; these peptides must be liberated from the proteins by specific enzymatic hydrolysis to exert their health effects. In this regard, it would be interesting to establish the sustained survival of yogurt bacteria in the presence of *A. sativum* that causes enhancement of milk protein proteolysis favouring the liberation of ACE inhibitory peptides at higher rate than that in control yogurt during the storage period.

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#### 6. Conflict of interest

The Author declares that there is no conflict of interest.

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