CHAPTER III

MATERIALS AND METHODS

3.1 Green tea

Commercially available green teas were purchased from local shops in the form of ground dried tea leaves in tea bags (BOH and OSK brands from Malaysia and Japan respectively). Both green teas had a similar method of preparation except that BOH green tea (MGT) was exposed to solar (1-2 h) and indoor (4 h) withering followed by, steaming, rolling and heat (90 °C) drying (BOH Company) as opposed to withering and steaming for OSK green tea (JGT) prior to rolling and heat (90 °C) drying (OSK Company).

3.2 Milk and yogurt bacteria

Fresh, pasteurized and homogenized cows' milk (4% full cream, as general consumer preference) was purchased from local shops. Nutritional value of present milk is shown in Table 3.1. Yogurt make from mixture of yogurt bacteria (Chris Hansen, Denmark) consisted of the following bacteria: *Lactobacillus acidophilus* LA-5,

Bifidobacterium Bb-12, *Lactobacillus casei* LC-01 and *Streptoccus thermophilus* Th-4 at the ratio of 4:4:1:1 and a capsule of probiotic mix containing L. bulgaricus, L. rhamnosus, B. infantis and B. longum in the ratio of (1:1:1:1) (Bio-Life, Malaysia).

Table3.1 Nutritional profiles (per 100 ml serving) of (4% full cream) milk

Average composition	Per 100 mL
Energy	62 kcal
Protein	3.2g
Carbohydrate	4.8g
Fat	3.3g
Calcium	135mg
Zinc	0.4mg
Vitamin A	160µg
Vitamin D3	3.0 µg
Vitamin B2	0.13mg

Nutritional Information presented is as stated on the product label

3.3 Preparation of starter culture

Yogurt starter culture was prepared by inoculating 1 L pasteurized full cream (4%) milk with yogurt bacteria (1% w/v). The milk-yogurt bacteria mixture was incubated at 41˚C for 12 h and the yogurt formed was stored at 4 ˚C and used as starter culture within 1 week. We routinely found the pH of the starter culture to range between 4.1 - 4.3 and viable bacteria to range between 2.0 - 5.0 × 10^6 cfu mL^{-1} and 6.0 -10.0 × 10^8 cfu mL^{-1} for

Lactobacillus spp. and *S. thermophilus* respectively on the 14 day of storage, which were slightly lower than those reported by Buyong, Kok, & Luchansky (1998).

3.4 Preparation of green tea infusions

Tea extracts were prepared essentially as described by Jaziri, Ben Slama, Mhadhbi, Urdaci, & Hamdi (2009). Ground tea (2 g) from each tea bag was taken out and infused in 100 ml of hot water (87-90 ˚C, 10 min, 2.0% (w/v) corresponding to the strength of a normal ‘‘cup of tea”). The infusate, filtered through Whatman No. 4 paper, was used for analysis.

3.5 Preparation of plain and tea-yogurts

Homogenized and pasteurized full fat (4%) milk (1 L) was divided into three portions. The first two portions were subjected to infusate with green tea (MGT or JGT) at 2.0% (w/v). The teas were infused for 10 min and the milks were filtered through sterile cotton to strain off visible plant particles. The strained milks thus obtained were cooled to 45 ˚C and inoculated with 10% (v/v) yogurt starter culture and aliquoted equally into three sterile beakers. The inoculated milk was incubated at 42 ˚C until the pH reached 4.5 to yield MGT-yogurt (MGTY) and JGT-yogurt (JGTY). Plain yogurt (PY) was made by inoculating the third milk portion without green tea infusion with 10% (v/v) yogurt starter culture followed by incubation at 42 ˚C until the pH reached 4.5. All yogurts were stored in a refrigerator (4˚C) and stored up to 28 days (Amirdivani & Baba, 2011).

3.6 Preparation of yogurt extracts

Plain- and green tea-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 (0.1M). The yogurts were then heated in a water bath (45°C) for 10 min prior to centrifugation (5000g, 10 min, 4°C). The supernatants were harvested and NaOH (0.1M) was added to adjust the pH to 7.0. The yogurt water extracts were re-centrifuged (5000g, 10 min 4 °C) and the supernatants were harvested and stored in a -20 °C freezer until required for analysis (Amirdivani & Baba,2011).

3.7 pH and titratable acid (TA) determination

Yogurt was initially homogenized in distilled water (1: 1 ratio) prior to pH determination (Kailasapathy, 2006) by using pH meter (model Mettler-Toledo 320, Shanghai) was determined by titration using 0.1 N NaOH. Adequately stirred yogurt (1 ml) was transferred into an Ehrlenmeyer flask containing 9 ml dH_2O. Several (3-5) drops of 0.1% phenolphthalein as pH indicator was added. The yogurt mixture was then titrated with 0.1N NaOH under continuous stirring until the development of a consistent pink color. The amount of acid produced during fermentation was calculated as follows:

$$\text{Lactic acid (\%)} = \text{Dilution factor (10)} \times V_{NaOH} \times 0.1\ \text{N} \times 0.009 \times 100\%$$

where V is volume of NaOH required to neutralize the acid

3.8 Total phenolic content (TPC) analysis

Total phenolic compounds were determined as described by Shetty, Vattem, & Clydesdale (2005). Briefly, 1 ml of tea yogurt extract was transferred into a test tube followed by the addition of 1 ml of 95% ethanol and 5 ml of dH_2O. Folin-Ciocalteu reagent (diluted 1:1 with distilled water) was added to each sample followed by thorough mixing using a vortexer. Na_2CO_3 (5%, 1 ml) was added to the reaction mixture and these were left

to stand for 60 min at room temperature. The absorbance (725 nm, Spectronic-Genesys, USA) values were converted to total phenolics expressed in microgram equivalents of gallic acid (µgGAE) per ml of the sample. A standard curve (see Figure 3.1) was established using various concentration of gallic acid (5-60 µg/ml) in methanol and these were included in the assay every time TPC analysis was carried out.

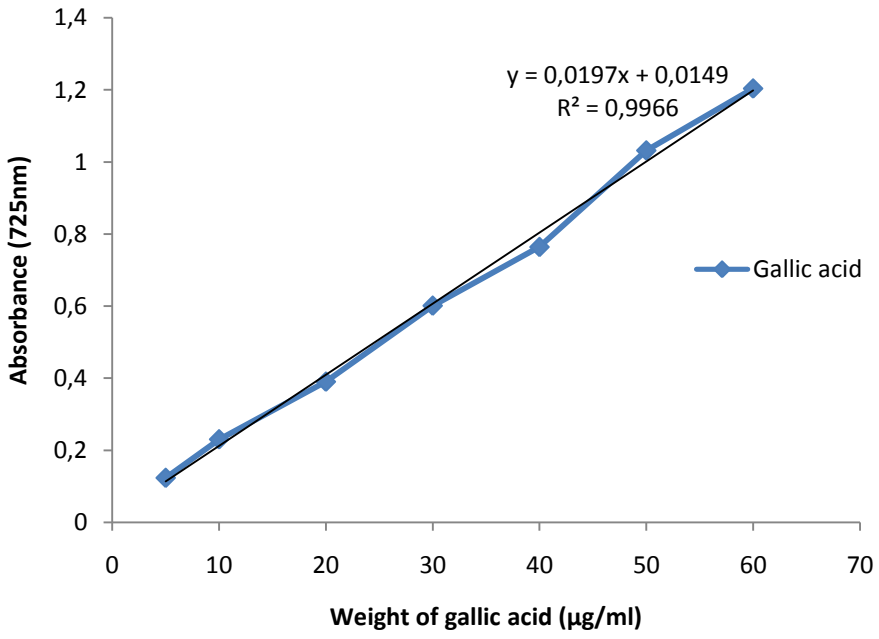

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Figure 3.1 Typical standard curve of gallic acid for the estimation of total phenolic content in yogurts

3.9 Determination of antioxidant activity

3.9.1 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical inhibition (DRI) assay

Yogurt water extracts (250 ml) were added into 3 ml of 60 mmol/L DPPH in ethanol (Sigma Aldrich, Germany). The mixture was left in the dark for 30 min. The decrease in

absorbance (517nm, Spectronic-Genesys, USA) was monitored until a constant reading was obtained. The constant reading for the yogurt extracts and control (consisting of 250 ml of water in place of extract) was used in calculating the % inhibition of DPPH oxidation (Apostolidis, Kwon, & Shetty, 2007) as follows:

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

3.9.2 Ferric reducing/antioxidant power (FRAP)

The ferric reducing antioxidant power (FRAP) was assessed according to methods described by Lucas et al. (2006). Briefly, appropriately diluted 400 µl of yogurt samples were mixed with 3.6 ml of the freshly prepared ferric-tripyridyltriazine (TPTZ) reagent. This buffer was prepared by mixing 300 mmol/L acetate buffer (pH 3.6), 8 mmol 2,4,6- tris (2-pyridyl) -s-triazine in 30 mmol/ L HCl; 20 mmol/L $FeCl_3$ in the ratio of 10:1:1. The mixtures were incubated at 37˚C for 10 min followed by brief centrifugation (1400 x g, 2 min) followed by absorbance reading (593 nm, Thermo spectronic 10 UV, (190-1100 nm)) of the blue TPTZ complex formed with reduced ferrous ions against a blank sample. The results were calculated from a standard scale of $FeSO_4.7H_2O$ and expressed as mmol/L (Najgebauer-Lejko, Sady, Grega, & Walczycka, 2011).

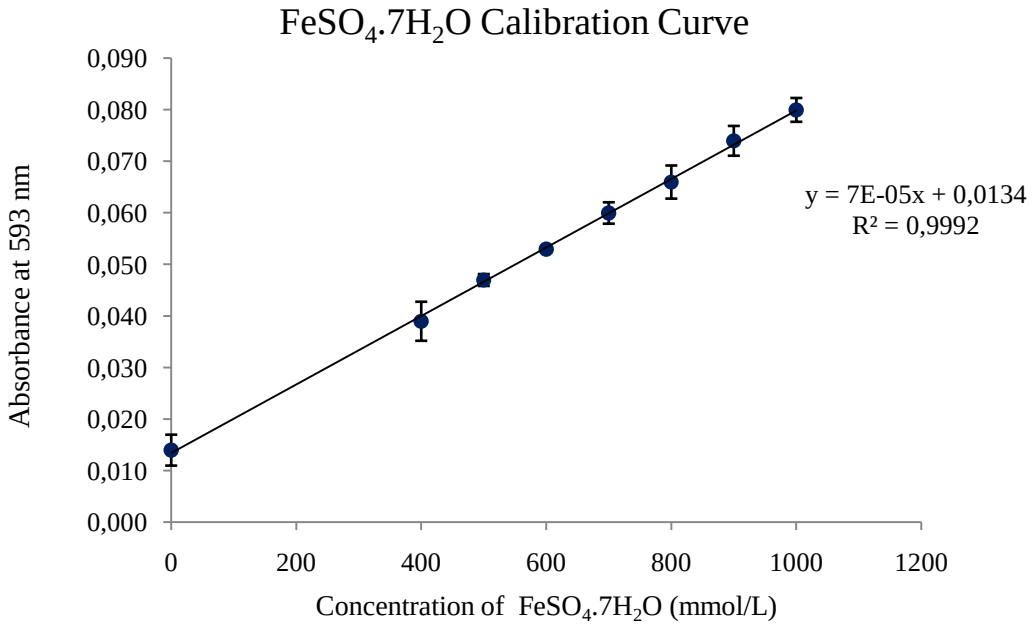

Figure 3.2 Calibration curve of $FeSO_4 7H_2O$ for the estimation of Ferric reducing/antioxidant power (FRAP) in yogurts

3.10 Proteolytic activity by o-Phthalaldehyde (OPA) method

To assay proteolysis of milk protein during fermentation, a small aliquot of yogurt extract (Church, Swaisgood, Porter, & Catignani, 1983) was added directly to 1.0 ml of OPA reagent in a 1.5 ml cuvette. The cuvette was inverted several times and then left for incubation for 2 minutes at room temperature. The absorbance (340 nm, Spectronic-Genesys, USA) was read and the peptide concentration was estimated against a tryptone standard curve (see section 3.10.2).

3.10.1 Preparation of OPA reagent

The OPA solution was made by combining the following reagents: 25 ml of 100 mM sodium tetraborate, 2.5 ml of 20% (wt/wt) sodium dodecyl-sulphate (SDS), OPA (40mg dissolved in 1 ml of methanol) and 100 µl of β-mercaptoethanol. The final volume

was made up to 50 ml using dH2O. OPA reagent is light-sensitive and thus must be protected from light sources during preparation and during the assay. This reagent was prepared fresh and used within 2 hours of preparation.

3.10.2 Tryptone standard curve

A standard curve of peptide concentration was prepared using tryptone. Various concentrations of tryptone standards (0.25, 0.5, 0.75, 1.00, 1.25, 1.50 mg/ml) were prepared from the stock solution. The tryptone standards were prepared and treated in the same manner for samples for each OPA assays. The resulting liner regression between absorbance (340nm) and trypton standard (mg/g) was constructed and used in the estimation of OPA peptides in sample.

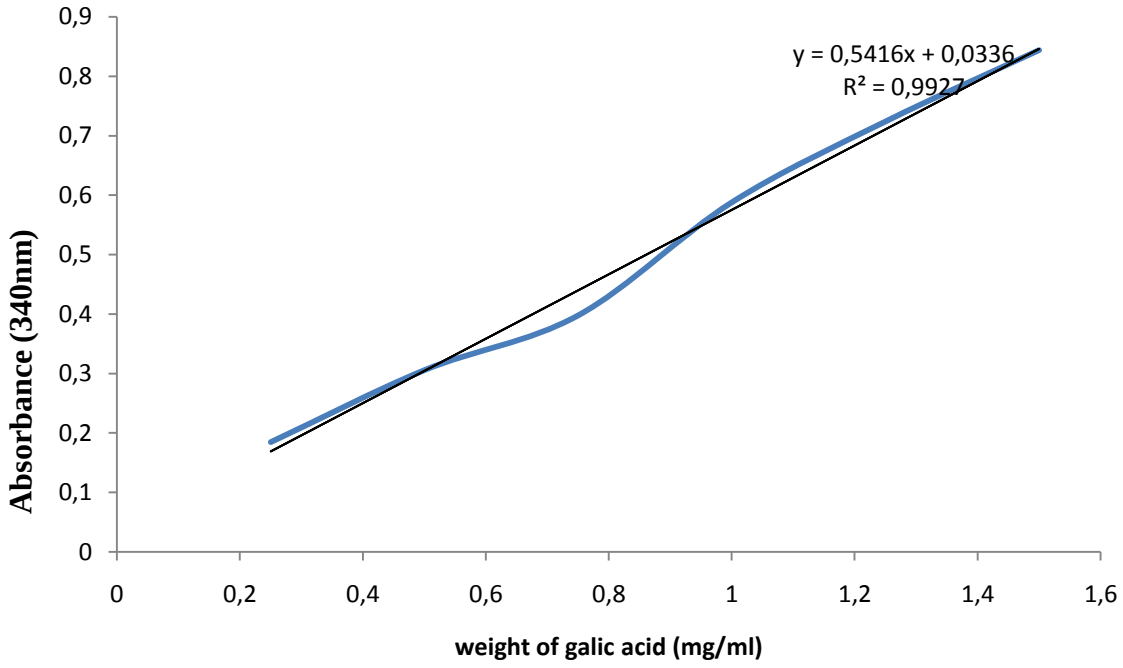

Figure 3.3. Typical standard curve of tryptone for OPA peptides (mg/g) in the O-Phthalaldehyde (OPA) method

3.11 Exopolysaccharide (EPS) isolation and estimation

EPS was isolated according to Amatayakul, Halmos, Sherkat, & Shah (2006). Yogurt was initially diluted with dH_2O in 1: 1 ratio. Proteins in the diluted samples were precipitated by adding 4ml of 20% (w/v) trichloroacetic acid (TCA) and these were separated by centrifugation (3500g for 30 min at 4°C). The supernatant was harvested and the pH was adjusted to 6.80 using 40% (w/v) NaOH, followed by boiling at 100°C for 30 minutes to denature whey proteins. After boiling, the solution was subjected to another centrifugation (3500g, 30 min, and 4°C) to separate denatured whey proteins. The supernatant was transferred into test tubes and was mixed with equal volume of cold absolute ethanol (99% ethanol) to precipitate the carbohydrate from supernatant. The precipitation was carried out overnight at 4°C and the suspension formed was separated by centrifugation (3500g, 30 min, and 4°C). The carbohydrate pellet was completely dissolved in 10ml of dH_2O or Milli-Q water and resultant suspension was subjected to sonication for 1 hour at room temperature. The suspensions were individually dialyzed at 4°C in dialysis membrane tubes with molecular weight cut-off 13,000 Da against tap water over 2 weeks period. Water was changed once in two days. The EPS concentration was quantified by using the phenol-sulphuric method and was expressed as glucose equivalent.

3.11.1 Phenol-sulfuric method

The phenol-sulphuric acid assay was based on the absorbance of 490nm of a coloured aromatic complex formed between phenol and the carbohydrate which determined reducing and non- reducing sugars and carbohydrate. This assay was used to prepare the glucose standard curve and the unknown amount carbohydrate present in the isolated EPS was determined by comparison with a calibration curve using a spectrophotometer.

Glucose (1.0g) was dissolved in 10ml of dH_2O to produce 100mg/ml stock solutions which were the aliquoted (500µl) and stored (-20°C) for later use. Thawed stock solution (10µl) was further diluted by mixing in 990µl dH_2O to yield glucose concentration of 1mg/ml. Known volumes from this solution were transferred into different test tubes and diluted so that the following final glucose concentrations were obtained: 0 (blank), 10, 20, 30, 40, 50, 80 and 100µg/ml. Each glucose standard (1.0ml) solution was thoroughly mixed with 500µl of 4% (v/v) phenol solution.

Concentrated sulphuric acid (98%, 2.5ml) was carefully added drop by drop using Pasteur pipette into glucose-phenol solution and the mixture was then thoroughly vortexed. The glucose-phenol-sulphuric acid mixtures were allowed to cool down to room temperature and the absorbance at 490nm was read after 15 minutes (Amatayakul, Halmos, Sherkat, & Shah ,2006).

3.12 Determination of enzyme activity

3.12.1 α-Amylase inhibition assay

3.12.1.a Amylase enzyme solution

Porcine pancreatic α-amylase was purchased from Sigma Chemical Company. According to the manufacture product description, one unit of enzyme will liberate 1.0 mg of maltose from starch per minute at pH 7.0 at 20°C. The enzyme concentration used in assay was 0.5 mg/ml (Apostolidis,Kwon & Shetty, 2007). Enzyme powder was dissolved in pre-chilled 0.02 M sodium phosphates buffer, pH 6.9 with 0.006 M sodium chloride, yielding a clear to hazy solution. This is due to the pressure of enzyme carriers, lactose which are partially soluble in the chilled buffer. The enzyme solution was prepared freshly and stored at temperature below 4°C prior to assay.

3.12.1.b Sodium phosphate buffer (0.02 M), pH 6.9 with .006 M sodium chloride

The following three solutions were prepared separately.

200 ml dH_2O was added to 1.582 of Na_2HPO_4

200 ml dH_2O was added to 1.062 of Na_2PO_4

100 ml dH_2O was added to 0.3506 g of NaCl

All 3 solutions were then mixed well followed by the addition of 400 ml dH_2O as to obtain the desirable pH of 6.90. If the pH deviated from 6.9, the pH was adjusted by adding either Na_2HPO_4 as base or NaH_2PO_4 as acid. Finally the solution was brought up to the final volume of 1000 ml in volumetric flask. The buffer prepared was stored at 25°C and used within 2 weeks (Apostolidis et al., 2007).

3.12.1.c Dinitrosalicyclic acid (DNSA) reagent

The original DNSA reagent developed by Miller, Lorenz (1959). Contained 0.63% DNSA, 18% titrate, 0.5% phenol, 0.5% sodium bisulfite, and 14% NaOH. A modified DNSA reagent used in this study reagent was prepared by dissolving (constant stirring) 2% (w/v) of DNSA with 1% NaOH. The other component of the modified DNSA reagent, the 18.2 % (w/v) potassium sodium tartrate, also known as Rochelle salts, was prepared separately using dH_2O. The reagent was prepared freshly prior to assay. Precautionary steps were taken as carbon dioxide tends to interfere the stability of reagent. In addition this color reagent should be protected from all light sources.

3.12.1.d 1% starch solution

Soluble starch (1g) was dissolved in 100 ml of sodium phosphate buffer. Constant

stirring at 90°C helped the dissolution of starch in buffer. The starch solution was then cooled and stored at 4°C. The starch solution was incubated at 25°C for 5 minutes prior to assay.

3.12.1.e α-Amylase inhibition assay

The α-amylase inhibition assay was adapted from Shetty, Secnik, & Oglesby (2005). Yogurt extracts (500µl) were mixed in 500µl of 0.02 M sodium phosphate buffer, pH 6.9 with 0.006 M sodium chloride containing 0.5 mg/ml. The α-amylase solution was incubated at 25°C for 10 minutes. After pre-incubation, 500 µl of a 1% starch solution in 0.02 M sodium phosphate buffer, pH 6.9 with 0.06 M sodium chloride was added to each tube at predetermined time intervals. The reaction mixtures were then incubated at 25°C for 10 minutes. The reaction was stopped using 1.0 ml of dinitrosalicyclic acid (DNSA) color reagent. The test tubes were then incubated in a boiling water bath for 7 minutes. Subsequently, 1.0 ml of 18.2 % tartrate solution was added to the each tube after the boiling session, before cooling to room temperature. The reaction mixture was then diluted after adding 10 ml of dH_2O. Absorbance was measured at 540 nm. The reading was compared to a control which is buffer solution in place of the extract .The formula used to calculate enzyme inhibition is stated as follows:

$$\text{Inhibition percentage} = \left(\frac{\text{Absorbance of control} - \text{Absorbance of extract}}{\text{Absorbance of control}}\right) \times 100$$

3.12.2 α--Glucosidase inhibition

3.12.2.a α-Glucosidase enzyme solution

α-glucosidase (1000U) was dissolved well in 3960µl (3.96 ml) of 0.1M potassium phosphate buffer (pH 6.90) and aliquoted into 33 ampoules and stored at -20°C. Each ampoule contained 30U/120µl of α-glucosidase enzyme solution (Apostolidis et al., 2007).

3.12.2.b 0.1M potassium phosphate buffer (pH 6.90)

The following two solutions were prepared separately:

200 ml dH_2O was added to 9.11 g K_2HPO_4

200 ml dH_2O was added to 6.49 g KH_2PO_4

Both 2 solutions were then mixed well followed by the addition of 400ml dH_2O as to obtain the desirable pH of 6.90. When pH deviated from 6.90, it was adjusted by adding either K_2HPO_4 as base or KH_2PO_4 as acid. Finally the solution was brought up to a final volume of 1000 ml in volumetric flask. The buffer prepared was stored at 25°C and used within 2 weeks.

3.12.2.c 5mM p-nitrophenyl-α-D-glucopyranoside substrate solution

Potassium phosphate buffer (0.1M, pH 6.90) was slowly added into 5mM p-nitrophenyl-α-D-glucopyranoside until it fully dissolved. This solution was freshly prepared prior to running α-glucosidase assay.

3.12.2.d α-Glucosidase inhibition assay

The α-glucosidase inhibition assay was performed essentially as described by Apostolidis, Kwon, & Shetty (2006). The reaction mixture contained 500µl of sample

extract and 1ml of 0.1M potassium phosphate buffer (pH 6.90) containing α-glucosidase solution (1.0 U/ml) and was incubated in a water bath at 25°C for 10 minutes. p-nitrophenyl-α-D-glucopyranoside solution (500 µl, 5 mM in 0.1 M potassium phosphate buffer (pH 6.9)) was then added to each tube at predetermined time intervals. The reaction mixture was further incubated at 25°C for 5 minutes. Absorbance reading was measured spectrophotometrically at 405 nm (Thermo spectronic 10 UV, (190-1100 nm)), before and after incubation period. The reading was compared to a control which had 500 µl of buffer solution in place of the extract. The α-glucosidase inhibitory activity was expressed as inhibition % as follows:

$$\text{Inhibition percentage} = \frac{\text{Absorbance of control} - \text{Absorbance of extract}}{\text{Absorbance of control}} \times 100$$

3.12.3 Calculation of IC50 for enzyme inhibition activity

Enzyme inhibition was expressed as the concentration of inhibitory compound that inhibition 50 % of enzyme activity (IC50), assuming that the activity of the blank is 100%. For enzyme inhibition studies 3 different strength of yogurt extracts (300 µl, 150 µl, 75 µl; dH_2O was used to make up the volume to 300µl) were determined. For α-amylase and α-glucosidase inhibition study; 3 different strength of yogurt extracts (500µl, 250µl and 125µl; dH_2O was used to make up the volume to 500µl) were determined. IC_{50} values were calculated by subjecting the data to a nonlinear adjustment programme (PRISM version 4.02 for Windows (GraphPad Software, Inc. San Diego, CA, USA) which estimates the value of the IC_{50} together with the standard error (Apostolidis et al., 2007).

3.13 Rheology measurements

The viscoelastic properties of the yogurt samples were determined by small amplitude oscillatory measurement (SAOM) using Bohlin CVO-R rheometer (Malvern Instrument UK). The rheometer was equipped with a temperature and moisture regulating hood and cone-plate geometry (20.0 ± 0.1°C, Cone type: 40/40 mm cone Plate and geometry gap of 0.150 mm). The temperature of the system was regulated by a viscotherm VT2 circulating bath and controlled (-40 to 180^{o}C, Peltier Plate System, Bohlin Instrument Ltd.) acts as temperature controller. The data of the rheological measurements were analysed with the supporting software. All the samples were gently stirred with a plastic spoon prior to loading a portion of the sample on the inset plate. The samples were subjected to a frequency sweep test using a frequency ramp from 0.001 to 10 Hz at a controlled strain mode ascertain the viscoelastic properties (Sendra, et al 2010).

3.13.1 Oscillation measurement

Amplitude sweep was first performed at a controlled strain mode with applied strain at a fix unit of range 0.0005 to 0.1 at a constant frequency of 0.5Hz.

3.13.2 Viscometry measurement

Viscometry measurement was performed by controlled shear rate from 0.01-80s^{-1}. Viscosity was measured in an increasing shear rate manner. All measurements were performed on yogurts subjected to 1, 7, 14, 21 and 28 days of refrigerated (4°C) storage.

3.14 Syneresis

Syneresis was carried out using siphon method as described by Amatayakul,

Sherkat, & Shah (2006). A cup of yogurt was weighed correct to 4 decimal places and the container was then positioned at an angle of 45° for 2 hours at 4°C. The whey accumulated was removed from the yogurt surface by using a syringe and the cups were then re-weighed. Syneresis was reported in terms of the percentage of whey lost using the following formula:

$$\text{Syneresis (\%)} = (\text{whey lost} / \text{sample weight}) \times 100\%$$

3.14.1 Water holding capacity (WHC)

Water hold capacity was performed as described by Minto ,(2010). The milk-mixture aliquots (30ml) were placed in pre-weighed 50ml centrifuge tubes ($Wt_{\textbf{empty tubes}}$; Oak Ridge Centrifuge Tubes). The tubes were then placed in an incubator to allow yogurt fermentation 41°C to occur until it reached pH4.5. The changes in pH were monitored using tracer container, i.e. another test tube containing milk with the same treatment conditions. The test tubes containing yogurts were weighed (Wt_{sample}) and subsequently placed in the refrigerator (4°C) followed by centrifugation (9500 rpm, 40 minutes, 10°C) the next day. Separated supernatant was discarded and tubes were reweighed (Wt_{pellet}). The weight of the pellet was determined by weight differences. Water holding capacity was reported in terms of percentage of pellet weight by using the following formula:

$$\text{WHC (\%)} = [(Wt_{pellet} - Wt_{empty\ tubes}) / (Wt_{sample} - Wt_{empty\ tubes})] \times 100\%$$

3.14.2 Total solid

The total solid was determined as described by Hooi et al. (2004). Approximately 3g of yogurt samples were placed in pre-weighed aluminum pans ($Wt_{empty\ pan}$) and these were weighed ($Wt_{before\ drying}$) and subsequently placed into atmospheric oven at 100°C for 5 hours drying. The dried samples were cooled down to room temperature in a desiccator containing cobalt (II) chloride anhydrous. The aluminum pans containing dried yogurt samples were re-weighed ($Wt_{after\ drying}$) and the total solid was reported in terms of percentage of yogurt solids using the following formula:

$$\text{Total solids (\%)} = [\ (Wt_{after\ drying} - Wt_{empty\ pan})/\ (Wt_{before\ drying} - Wt_{empty\ pan})\ ]\ x100\%$$

3.15 Microbial assay

3.15.1 Enumeration of viable cell (VCC) in yogurt

Yogurt bacteria were enumerated using spread plate method and pour plate method for *S. thermophillus* and *Lactobacillus* spp. respectively. The pour plate method utilised MRS (De Man, Rogosa and Sharp) agar medium to support the growth of *Lactobacillus* spp. under anaerobic condition whereas the spread plate method used M17 agar medium to support the growth of *S. thermophilus.* Samples of yogurts were diluted to 10^{-4}, 10^{-5} and 10^{-6} in sterile peptone water and 1.0 ml or 0.1ml aliquot of the diluted yogurt was plated on individual MRS or M17 plate respectively (Kailasapathy et al. 2008).

3.15.2 MRS agar preparation

MRS agar (62g) was properly suspended in 1L distilled water. The mixture was dissolved by mild heating (45°C) with frequent agitation followed by boiling for one minute. The agar was sterilized by autoclaving (121°C for 15 min) followed by cooling to

45-50ºC prior to pouring (15ml) into Petri dishes.

3.15.3 M17 agar preparation

M17 agar is a nutritionally rich medium used for the cultivation and enumeration of fastidious *lactic streptococci* (Davis, Underwood, & Gasson, 1981). M17 agar (55g) was resuspended in 1L dH_2O with mild heating (45°C) and frequent agitation. The solution was then boiled for one minute and then sterilized by autoclaving (120°C, 15 min) followed by cooling to 45-50ºC. The molten M17 agar (15ml) was then transferred into petri dishes. Lactose (10g) was dissolved in 100ml of dH_2O to produce 10% (w/v) lactose solution and this was sterilized by autoclaving. The sterilized lactose solution was later added into the M17 medium and the mixture was mixed thoroughly.

3.15.4 Preparation of peptone water buffer

Buffered peptone water (20g) was added into 1L dH_2O followed by thorough stirring to assist mixing. The solution was distributed into different 15ml-centrifuged tubes and was then sterilized by autoclaving at 121°C for 15 minutes. Samples were taken at predetermined sampling times, i.e. after 1, 7, 14, 21 and 28 days of refrigerated storage. Yogurt samples were vortex-mixed (Stuart Scientific, UK)) with 9 ml of buffered peptone water (Oxoid, UK). The diluted yogurt samples were then decimally (1:10) diluted to 10^{-7} using peptone water as the diluents. Empty petri dishes were inoculated with 1.0 ml of diluted yogurt followed by the addition of 15 ml of melted (45˚C) MRS agar into the petri dish and the content was mixed thoroughly by gentle tilting and swirling. The dishes were inverted once the agar has solidified, and incubated anaerobically (Revco Ultima, USA). Parafilm was used to seal the petri dishes to prevent the entry of air because *Lactobacillus*

spp. are anaerobic bacteria. The diluted yogurt (0.1 ml) was spread evenly on the agar and incubation was carried out at 37˚C for 24-48 hours. Colonies formed on agar were counted and colony forming unit (CFU) per ml or per g was calculated as follows:

CFU/ mL = CFU/plate x dilution factor

*CFU= colony forming unit

3.16 Determination of organic acids

3.16.1 Analysis of organic acid

Sample preparing of organic acids which were analyzed by liquid chromatography mass spectrophotometer (LC-MS) using the method by Marsili, Ostapenko, Simmons, & Green (1981) as described by Narvhus, Osteraas, Mutukumira, & Abrahamsen (1998) with modification. The characteristic of LC-MS used in this assay is explain in section 3.17.A volume of yogurt sample (1.0 ml) was mixed with 0.2 ml 0.5 M H_2SO_4 and 8.0 ml acetonitrile (HPLC grade) followed by centrifugation (6000 g, 10 min, 4 ˚C). The supernatant was passed through 0.2 µm nylon filters (Sartorius, Goettingen, Germany). A volume of 20 µl of the filtered sample was injected into the column. Concentration of each organic acid was expressed as mg mL^{-1}. The

3.17 LC-MS analysis of phenolic compounds and organic acids

LC-MS is a chemistry technique that utilizes liquid chromatography capability to 'physically separate compound' and mass spectrometry capability to 'analyse the mass of separated compounds'. This is a powerful technique used for the general detection and

potential identification of chemicals in the presence of other chemicals (in a complex mixture) and it has very high sensitivity and selectivity. The LC-MS/MS equipment consisted of a quaternary pump with vacuum degasser, an auto injector (Agilent Technologies, Santa Clara, California, USA) and a mass spectrometer (AB Sciex 3200QTrap, Toronto, Canada). Chromatogram was recorded with a full scan and MS/MS data collection and negative electrospray ionization. Data collection and subsequent processing were performed using AB Sciex Analyst software. The analytical column (C18, 50mm x 1.1 mm i.d; Phenomenex Aqua, USA) was operated at 35˚C. The mobile phase A was water with 5mM ammonium formate and 0.1% formic acid, while mobile phase B was acetonitrile with 5mM ammonium formate and 0.1% formic acid. The chromatographic program run consisted of a linear gradient step of 10% B to 90% B in 7.0 min followed by isocratic hold for 3.0 min and re-equilibration back to 10%B for 5.0 min. Total run time was 15.0 min with effective run time for organic acids analysis being 8.0 min. The chromatographic program run consisted of a linear gradient step of 10% B to 90% B in 4 min, holding period of 2 min, followed by linear gradient back to 10% B and reequilibrate in 2 min. The column temperature was maintained at 40°C throughout the chromatographic run.

3.18 Sensory analysis

The consumer acceptability studies was carried out using organeoleptic evaluation of yogurt by a jury of 10 panelists (mean age 25 years old). Six parameters i.e. flavour, appearance, colour, texture, aroma and overall appreciation were evaluated using a sensory rating scale of 1-10 (1 for extremely dislike, to 10 for extremely like; Larmond, 1987). The panels recognized the yogurt only by codes. Each panel was requested to rinse their mouth

by drinking mineral water after assessing each yogurt. Flavor was assessed by the estimation of acidity developed by specific lactic acid bacteria in the samples. A strict protocol was imposed to panelists to minimize variability. At each session, subjects tasted samples of chilled (4°C) yogurt (5 g). They were asked to keep the yogurt in the mouth for 12sec prior to swallowing. The subjects tasted samples of yogurt in the most natural possible way by keeping their mouth closed and swallowing the product. The yogurt samples were presented in random order. A small period of several minutes rest was required between sample tasting .

3.19 Statistical analysis

A total of three separate experiments were carried out and assays were performed in triplicates. Data were expressed as mean ± standard deviation and the data were analyzed using SPSS 19.0 (Chicago, IL, USA) for Windows. General Linear Model procedures and Tukey test for means comparison were used for determining significant difference at $p < 0.05$.

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