CHAPTER VI

EFFECTS OF GREEN TEA (*Camellia sinensis*) ON ANTIOXIDANT PROPERTIES OF YOGURT DURING STORAGE AS EVALUATED BY CHANGES IN POLYPHENOLIC COMPOUNDS CHARACTERISTICS BY LC-MS

Introduction

Fermented milk confers several health benefits attributed to the products of microbial fermentation of milk carbohydrate, fat and protein (Dhiman, MacQueen, & Luchini, 2001). The popularity of fermented dairy products, particularly yogurt, has encouraged the development of variety of new products enriched with probiotics, prebiotics, fruit and plant materials with the intention to increase the variety of dairy products with functional properties. When plant materials are used these include fibers (Sendra et al., 2008), phytosterols (Hansel et al., 2007), green and black teas (Jaziri, Ben Slama, Mhadhbi, Urdaci, & Hamdi, 2009), aqueous extract from vegetable (Karaaslan,

Ozden, Vardin, & Turkoglu, 2011) and aromatic plants (Amirdivani & Baba, 2011). The inclusion of these plants-based additives, naturally rich in phenolic compounds, antioxidants and organic acids, into yogurt may benefit the fermentation process, storage life, organoleptic and physicochemical properties (Zare, Champagne, Simpson, Orsat, & Boye, 2012).

The beneficial effects of tea by virtue of the phytochemical contents and antioxidant activities may include therapeutic effects against several chronic and degenerative diseases such as cancer, cardiovascular disorder, diabetes and obesity (Thielecke & Boschmann, 2006). Technically green tea is defined as heat-dried unfermented tea leaves and thus contains the natural and healthy elements of the fresh leaves. In Japan, the plucked leaves are quickly steamed on a bamboo tray over water or in a steaming machine. The leaves are then rolled by hand or machine before being heat-dried. However, in Malaysia the fresh tea leaves are normally spread out on bamboo trays and exposed to warm air for one to two hours prior to rolling and heat-drying. However, subtle differences in this drying process could lead to marked changes in the chemical and antioxidant properties of green teas (Lin, Tsai, Tsay, & Lin, 2003).

The most abundant compound in brewed green tea is a class of polyphenols known as catechins capable of scavenging superoxide anion and hydroxyl radicals, reducing lipid peroxyl radicals and inhibiting lipid peroxidation (Graham, 1992). Phenolic compounds also play important roles in the formation of sensory and dietary attributes of food products. The flavonoids and anthocyanins for instance may also confer higher antioxidant activity than vitamin C, vitamin E and β-carotene (Eberhardt, Lee, & Liu, 2000). Brewed green tea contains slightly fruity and greenish aroma notes (S. Lee, Park, Kim, & Kim, 2007) and thus the enrichment of foods with tea phenolic compounds is a sound strategy to produce

functional foods exhibiting higher antioxidant activity (Najgebauer-Lejko, Sady, Grega, & Walczycka, 2011). The aims of the present studies were;

1) To enrich yogurt with phenolic compounds using green tea-rich natural compounds to produce functional foods exhibiting high antioxidant activity

2) To determine the effects of green tea on yogurt acidification during storage at 4˚ C.

3) To determine the effects of refrigerated storage on changes in yogurt's antioxidant activities

4) To investigate the effects of green tea on the microbial production exopolysaccharide in yogurt

5) To determine effects of green tea on *in vitro* inhibition of α-glucosidase and α-amylase

6.1 Materials and Methods

6.1.1 Plant materials

The two different commercially available green teas (BOH and OSK brands from Malaysia and Japan respectively) were used in the present studies. Details on the processing steps in the production of these teas are as described in Section 3.1.

6.1.2 Milk and yogurt bacteria

Fresh, pasteurized and homogenized cows' milk and the organisms used are as described in Section 3.2.

6.1.3 Preparation of starter culture and tea infusion

The method of starter culture and tea preparation was determined as described in

Section 3.3 and 3.4.

6.1.4 Preparation of plain and tea-yogurts

The method of yogurts making is described in Section 3.5.

6.1.5 Preparation of yogurt extract

Extraction method of plain and tea yogurts was described in Section 3.6.

6.1.6 pH and titratable acid (TA) determination

The pH and TA of all samples were measured as indicated in Section 3.7. The reduction in pH readings was the difference between the observation on day 0 (fermentation time) and on day 28 of storage.

6.1.7 Total phenolic content analysis (TPC)

The TPC of samples was assessed as described in Section 3.8.

6.1.8 Determination of antioxidant activity

The DPPH inhibitory activity as well as FRAP value of samples was determined according to procedure described in Section 3.9.1 and 3.9.2 respectively.

6.1.9 Determination of viable lactic acid bacteria (LAB)

The method of enumeration of viable cell for all samples was described in Section 3.15.

6.1.10 Exopolysaccharide (EPS) isolation and estimation

The quantity of EPS in samples was measured during storage of 28 day at 4˚C under Section 3.11.

6.1.11 Proteolytic activity in yogurt by *o-Phthalaldehyde* (OPA) method

Proteolytic activity in yogurt was determined as described in Section 3.10

6.1.12 Determination of enzyme activity

Inhibition of α-amylase and α-glucosidase activity by yogurt was measured by the procedure described in Section 3.12.1 and 3.12.2.

6.1.13 LC-MS analysis of phenolic compounds

Extracted samples of yogurt were analysed for phenolic compounds using LC-MS as described in Section 3.17.

6.1.14 Statistical analysis

Statistical analysis of data obtained was performed as described in Section 3.19.

6.2 Results and discussion

6.2.1 Effects of green teas on the changes of acidification of yogurts during storage

The pH values and acidity of the yogurts containing green teas were measured during 4 week of storage and the results are shown in Table 6.1. Initial pH values for green tea and plain-yogurts were 4.52± 0.01, 4.51± 0.01 and 4.53± 0.03 for MGT, JGT and plain-yogurts respectively. Yogurts TA values ranged 0.88 – 0.96 % lactic acid equivalent on day

0 of storage (i.e. pH (4.5) at the point of incubation termination). Refrigerated storage for 28 days resulted in continued decrease in pH and increase in lactic acid production for all yogurts. Japanese green tea yogurt (JGTY) had the fastest rate of pH reduction (-0.085 pH unit/day) followed by Malaysian green tea yogurt (MGTY) and plain yogurt (PY) (-0.071 and -0.058 pH unit/day respectively). This resulted in the pH of JGTY being the lowest (pH 4.06) on day 28 of storage followed by MGTY and PY (pH 4.14 and 4.20 respectively). The rate of acid formation in green tea yogurts (-0.40 pH unit/hr and -0.42 pH unit/h for MGTY and JGTY respectively) was higher than that for plain-yogurt (-0.30 pH unit/h) during fermentation (See section 5.3.1). TA is the total amount of hydrogen ions present in the fermented milk sample with the exception of those bound to alkaline ions. Higher increase ($p>0.05$) of TA formation was shown in both green tea yogurts (1.13± 0.02 and 1.15± 0.02 % for MGTY and JGTY respectively) than plain yogurt (1.08±0.02 %) in 28 day of storage. Different phytochemical contents in the green teas may have influenced post-acidification to varying degree during storage in the same manner as the effects of fruits on yogurt during storage (Kailasapathy, Harmstorf, & Phillips, 2007). This could occur by the phytochemical modulation of growth and activity of *S. thermophilus* (Kailasapathy et al., 2007) and other LAB (Dave & Prajapati, 1994) which are responsible of pH decline during refrigerated storage.

Table 6.1 pH and titratable acid (as % lactic acid) of yogurts during storage

Storage Day	**pH**			**TA**		
	PY	MGTY	JGTY	PY	MGTY	JGTY
0	4.53± 0.03	4.52± 0.01	4.51± 0.01	0.88±0.06	0.91± 0.03	0.96± 0.02
7	4.40± 0.02	4.36± 0.04	4.31± 0.01	0.96±0.01	1.03± 0.03	1.02± 0.03
14	4.32± 0.01	4.27± 0.06	4.24± 0.01	1.03±0.02	1.05± 0.04	1.08± 0.02
21	4.28± 0.01	4.21± 0.06	4.16± 0.02	1.06±0.01	1.09± 0.03	1.12± 0.02
28	4.20± 0.04	4.14± 0.05	4.06± 0.01	1.08±0.02	1.13± 0.02	1.15± 0.02

pH profiles (unit/day) and changes in tritratable acid (% lactic acid) during the storage process MGTY=Malaysian green tea yogurt, JGTY=Japanese green tea yogurt, PY= plain yogurt. Data are presented as.mean ± SD (n=3)

6.2.2 Effects of green tea on viable cell count in yogurts during refrigerated storage

The changes in viable cell counts (VCC) of *Lactobacillus spp*. in yogurts are presented in Figure 6.1. Viable number of *Lactobacillus spp.* was highest in fresh MGT yogurt (12.45x 10^8cfu mL^{-1}) followed by JGT- and plain-yogurts (11.06 x 10^8 and 6.17 x 10^8cfu mL^{-1} respectively).The survival of *Lactobacillus spp.* decreased gradually ($p<0.05$) to 11.52 x 10^8 and 9.95 x 10^8 cfu mL^{-1} in MGT-and JGT-yogurts respectively by day 28 of storage.. PY (6.0 x 10^8 cfu mL^{-1}) showed the least changes in viable *Lactobacillus spp.* counts compared to fresh yogurt.

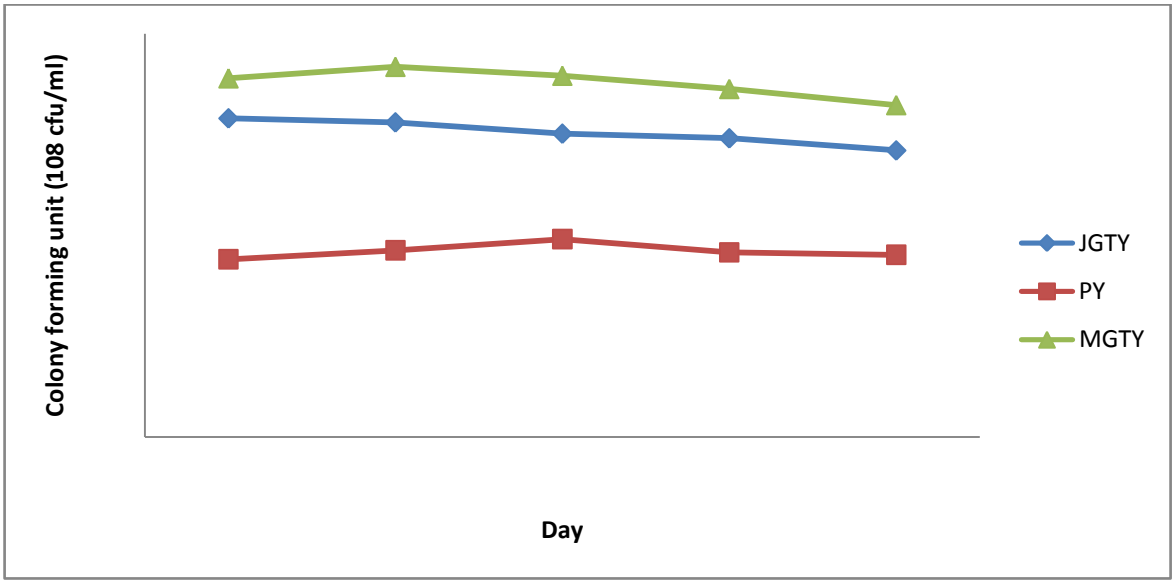

Figure 6.1 Viable *Lactobacillus spp.* count (10^8 cfu mL^{-1}) during storage period

VCC of S. *thermophilus* in yogurts are shown in Figure 6.2. VCC in fresh yogurts were marginally higher ($p<0.05$) in green tea-yogurts (119 x 10^6 and 121 x 10^6 cfu $mL^{-1}$for MGTY and JGTY respectively) than in PY (104 x 10^6 cfu mL^{-1}). All yogurts had increased viable *S. thermophilus* counts ($p<0.05$) on day 7 of storage in comparison to those in fresh yogurts with highest value shown by MGTY (138 x 10^6 cfu mL^{-1}) followed by JGTY and PY (129 x 10^6 and 110 x 10^6 cfu mL^{-1}respectively). Extended storage resulted in gradual decrease in viable *S. thermophillus* counts in all yogurts towards 90 x 10^6 cfu mL^{-1} by day 28 of storage.

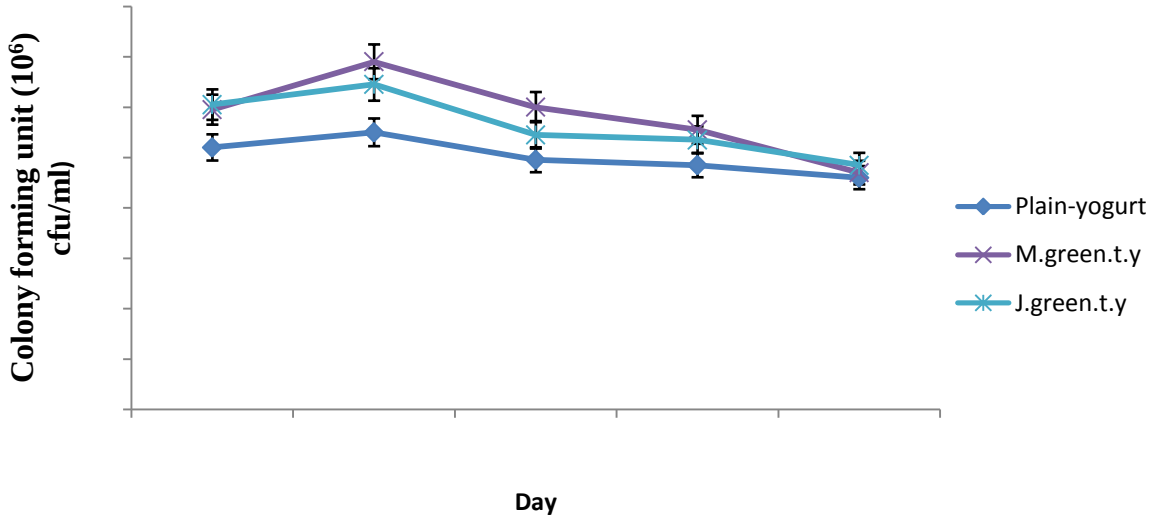

Figure 6.2 Viable *S. thermophilus spp*. count (10^6 cfu mL^{-1}) during storage period

A decline in yogurt bacteria survival in yogurt are affected by pH, hydrogen peroxide, dissolved oxygen in fermented milks (Lucas, Sodini, Monnet, Jolivet, & Corrieu, 2004), strain used, interaction between species present, culture conditions, chemical composition of the fermentation medium, availability of nutrients, growth promoters and inhibitors, level of inoculation, incubation temperature, fermentation time and storage temperature (R. I. Dave & Shah, 1996). Refrigerated storage resulted in significant losses in the cell numbers for both *Lactobacillus spp* and *S. thermophilus* and thus made yogurt having limited shelf life. Thus it is important in the study of improvement of any fermented food to monitor the changes in microbial growth as a result of new protocol in fermentation. Additional growth performance of these bacteria, either during fermentation or storage, may implicate increased therapeutic benefits such as modification of the immune system, reduction in cholesterol, alleviation from lactose intolerance and faster relief from diarrhea (Heller, 2001). *S. thermophilus* counts increased even during

refrigerated (4°C) storage and in green tea-yogurts could be due to residual microbial growth which was enhanced during fermentation at 41°C. The number of *Lactobacillus* species was found to decrease considerably more during storage than for *S. thermophilus*. Refrigeration increased viable *Lactobacillus spp* counts in plain yogurt till day 14 of storage, but the presence of green tea inhibited this increase. *Lactobacillus* is anaerobic bacteria (Talwalkar & Kailasapathy, 2004) and the incorporation of air into the yogurt during the mixing of green tea extract with milk at the preparation stage may be partially responsible in the initial reduction of viable cell counts. Thus additional care may need to be exercised to minimise the inclusion of oxygen in the milk. Hydrogen peroxide produced during manufacture and storage of yogurt (Gilliland & Speck, 1977) may also be used to explain the loss in viable *L. acidophilus*. Lower *Lactobacillus spp* .in green tea-yogurts may also be directly caused by the higher sensitivity these bacteria than *S. thermophilus* towards the presence of phytochemicals in the tea extracts (Talwalkar & Kailasapathy, 2004).

6.2.3 Effects of green tea on exopolysaccharides (EPS) content in yogurt during fermentation and refrigerated storage

Exopolysaccharides (EPS) are produced by lactic acid bacteria (LAB) during fermentation (Cerning, 1990) can act as natural bio thickeners. The EPS content during storage of yogurts is presented in Figure 6.3. The EPS in fresh yogurts was 202.3, 158.1 and 122.3 µg/ml for MGTY, JGTY and PY respectively, which are within the wide range of EPS production (50 to 2700 µg/ml) by *Lactobacillus spp.* (Macedo, Lacroix, Gardner, & Champagne, 2002). Refrigerator storage increased EPS content in 7 day for both green tea yogurts (244 and 170 µg/ml for MGTY and JGTY respectively) followed by gradually

decreased thereafter. Increased EPS content in yogurt was previously reported by Purwandari, Shah, & Vasiljevic (2007) although increased (Amatayakul, Sherkat, & Shah, 2006) or stable (Doleyres, Schaub, & Lacroix, 2005) EPS content in yogurt were also reported during 28 days refrigerated storage. In this regard the presence of green tea has positive attribute with respect to EPS production by yogurt bacteria because the lower EPS content in PY was unchanged this storage period. Increased EPS content in green tea-yogurts during the first 7 days of refrigerated storage could be due to the enhanced number of the EPS producer (*S. thermophilus* and *Lactobacillus spp*)(Aslım, Beyatli, & Yuksekdag, 2006). While the decrease in EPS content after day 7 could be attributed to the presence of enzymes capable of degrading EPS.

The relative bacterial contributions to total EPS production in the presence of green tea were not studied but it is anticipated that *Lactobacillus spp.* contributed more EPS than *S. thermophilus* (Aslim, Yu¨ksekdag, Beyatli, & Nazime Mercan, 2005). The lower bacterial count for *Lactobacillus* spp. and *S. thermophillus* in the plain yogurt compared with green tea yogurts (Figures 6.1 and 6.2) may collectively contribute to the lowered EPS production. This suggests that preferential growth stimulation of *Lactobacillus* spp over than *S. thermophilus* by green tea offers a potentially natural way of manipulating the production of EPS.

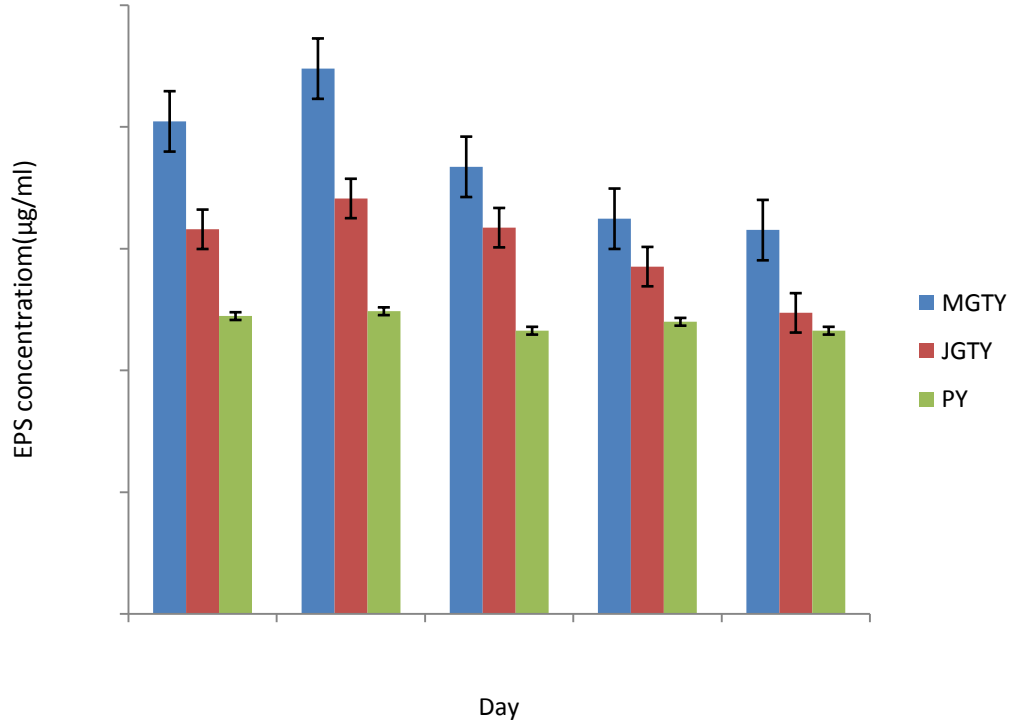

Figure 6.3 Changes of exoplysaccharide (EPS) content of yogurts during storage at 4˚C. MGTY=Malaysian green tea yogurt, JGTY=Japanese green tea yogurt, PY= plain yogurt. Data are presented as means ± SD (n=3)

6.2.4 Effects of green tea on proteolytic activity (OPA values) of yogurts during storage

The proteolytic activity, measured as change in absorbance at 340 nm (A_{340}) during storage is shown in Figure 6.4. The OPA-based spectrophotometric assay which quantities α-amino groups reflects the extent of proteolytic activity. The OPA values in fresh yogurts, were higher in the presence of green teas (22.01±2.48 and 18.58±1.55 mg/ml respectively for MGT and JGT yogurts) than in its absence (7.98 ± 1.2 mg/ml, $p<0.05$). All yogurts showed an increase in extent of proteolysis ($p<0.05$) by day 7 of storage (47.33± 1.3, 37.58± 0.9 and 14.20±0.72 for MGTY, JGTY and PY mg/ml respectively), followed by a consistent reduction until the 28th day of storage for MGTY, JGTY and PY (29.98±0.89,

14.87±1.1 and 5.13±1.0 mg/ml respectively). *L. acidophilus* and *S. thermophilus* are metabolically active even at 4 ˚C (Papadimitriou et al., 2007) and thus the extent of proteolysis by these bacteria could have been enhanced by the presence of green teas as shown by a substantial increase in OPA values in yogurt after the first 7 days of refrigerated storage (Figure 6.4). Differences in green tea-induced microbial proteolysis could lead to enhanced alteration of protein breakdown with potentially higher production of bioactive peptides (Shahidi & Zhong, 2008). This was demonstrated by the relatively unchanged OPA values for PY in contrast to reduced OPA values for green tea yogurts during day 7 and 21 of storage (Figure 6.4). Although a reduction in OPA values suggests a decrease in α-amino groups reacting with β-mercaptoethanol, thus lower absorption at 340 nm, it does not necessarily implicate significant utilization of amino acids by the yogurt bacteria. Degradation of amino acids to yield carbon skeletons that are used as nutrients by LAB (Amirdivani & Baba, 2011) is more likely to occur during fermentation at 41 ˚C rather than during storage at 4˚C. Thus the extent of proteolysis and the sizes of peptides produced from the fermentation are expected to be different in the presence of green tea and this need to be further studied in the future.

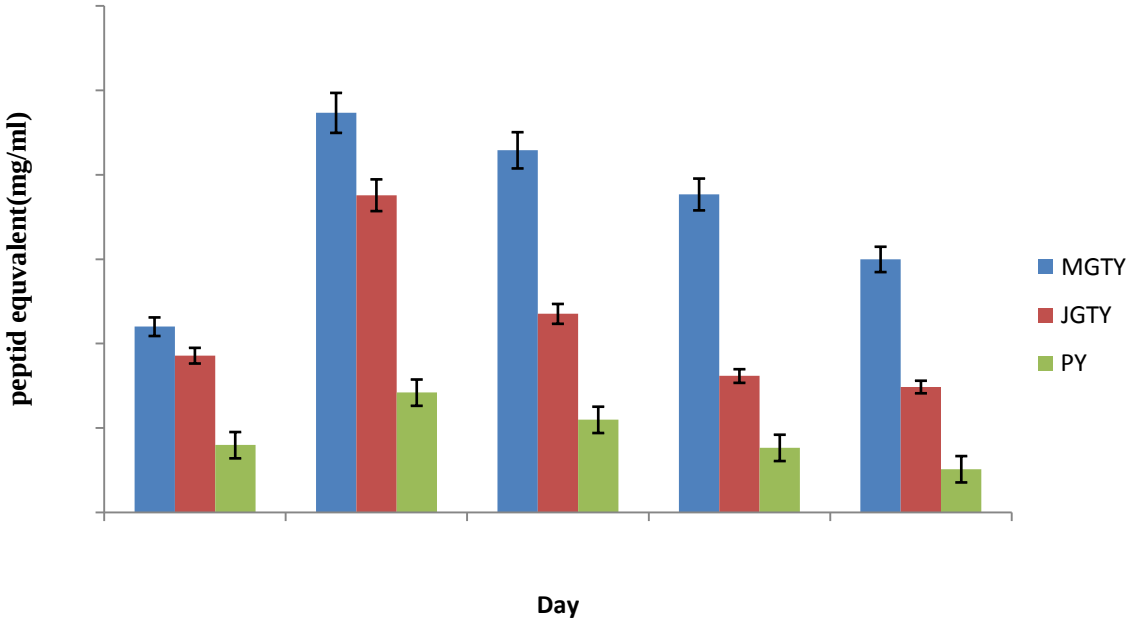

Figure. 6.4. Changes in o-phthalaldehyde (OPA) values in plain- and green-yogurts during refrigerated storage (4 ˚C).MGTY=Malaysian green tea yogurt, JGTY=Japanese green tea yogurt, PY= plain yogurt. Each experiment was repeated three times and values are means ± SD (n=3).

6.2.5 Effects of green tea on total phenolic content (TPC) of yogurts during storage

TPC in fresh yogurt was highest in MGTY (35.7 µgGAE/ml) followed by JGTY and plain-yogurt (25.03 and 7.32 µgGAE/ml, respectively; $p<0.05$, Table 6.2). Refrigerated storage increased ($p<0.05$) TPC for all yogurts on day 7 of storage (46.31± 1.30, 34.31± 1.08 and 11.87± 1.01 for MGT, JGT and plain-yogurts respectively), followed by a gradual decrease during the next 21 days. The TPC of all yogurts decreased significantly ($p < 0.05$) during the storage period and reached lower values than on day 7 of refrigerated storage.

Higher TPC in MGTY than in JGTY could be accounted by proportionately more phenolic compounds degraded by the solar process underwent by Malaysian green tea. The TPC value is complicated by the fact that even without green tea in PY, the TPC values established for PY (contributed by milk protein-derived phenolic compounds) may change

due to green tea-induced microbial degradation of milk protein in green-tea yogurts. This would add up to the differences in degradation of phenolic compounds derived from added green tea. Notwithstanding these uncertainties, most phenolic acids are powerful antioxidants capable of exerting antibacterial, antiviral, anti-carcinogenic, anti-inflammatory and vasodilatory actions (Duthie, Duthie, & Kyle, 2000). Therefore, the addition of green tea may thus be seen as a healthy way to increase TPC-derived antioxidant properties in yogurt.

Table 6.2 Total phenolic content of green tea and plain -yogurts during storage (4˚C)

Storage day	**JGTY**	**MGTY**	**PY**
0	25.03± 1.01[a]	35.7± 1.05[a]	7.32± 1.10[a]
7	34.31± 1.08[a]	46.31± 1.30[a]	11.87± 1.70[a]
14	33.28± 1.20[a]	45.45± 1.40[b]	10.55± 1.06[b]
21	31.77± 0.84[b]	39.07± 2.02[b]	10.20± 1.06[b]
28	29.16±1.07[b]	38.03± 1.08[b]	9.18± 1.02[b]

Total phenolic contents (TPC) in yogurt in the absence (PY) or presence of Malaysian (MGT) or Japanese (JGT) green tea yogurt was evaluated using FolineCiocalteu. Total phenols were expressed as ug gallic acid equivalent (ugGAE)/ml. Values are Mean ± SD (n = 3). [a, b] Different superscript within the same row of each sample differ significantly ($P<0.05$).

6.2.6 Effect of green tea extracts on the changes of antioxidant activity of yogurts during storage

5.3.6.a DPPH radical inhibition (DRI) assay

Free radical scavenging activity in fresh yogurts (Figure 6.5) were higher ($p<0.05$) for MGTY and JGTY (39.18±1.2 and 31.2±1.5% respectively) than for plain yogurt

(17.43±0.21%). Refrigerated storage resulted in increased antioxidant capacity during the first 7 days, with maximum values capacity recorded by MGTY (42.23± 1.5%) followed by JGTY and plain yogurt (37.11± 1.15 and 24.19± 2.01% respectively). However continue storage longer than 14 days resulted in reduced free radical scavenging activity for all yogurts (-0.64,-1.07 and – 0.5% for MGTY, JGTY and PY respectively). The free radical scavenging activity in yogurt can be attributed to products of milk fermentation as demonstrated by the 2 fold increase in antioxidant activity in PY compared to that in fresh milk (8.11± 0.23%). Enzymic degradation of macromolecules in milk is known to yield smaller molecules such as polypeptides and organic acids (Ercili-Cura et al., 2012) with bioactive (i.e. antioxidant) properties. This could also explain the further increase in free radical scavenging activity associated with post-acidification (Trigueros, Sayas-Barberá, Pérez-Álvarez, & Sendra, 2012) in yogurts by day 7 of storage.

The free radical scavenging activity of green tea phenolic compounds is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers (Perumalla & Hettiarachchy, 2011). The present studies showed that the magnitude of increase in free radical scavenging activity during these two periods (fermentation of milk and post-acidification) cannot be explained by the presence of green tea alone (Figure 6.5).This suggests that there were enhancement effects of green tea on microbial enzymic activities resulting in increased formation of bioactive compounds. The exact nature of the responsible compounds was not further investigated in the present studies but could be most likely attributed to increased proteolysis (increased OPA values; Figure 6.4) and organic acid formation (increased TA; Table 6.1). A practical application from the present finding is that the consumption of yogurt is highly recommended about 7 days after yogurt-making in order to benefit from the high VCC yogurt bacteria contents

(Van de Water, Keen, & Gershwin, 1999; Figure 6.2) and free radical scavenging activity therapeutically beneficial for protective cardiovascular effect. The reduction in free radical scavenging activity after the 14 days of refrigerated storage of yogurt has been previously described (Amirdivani & Baba, 2011) due to increased degradation of phenolic compounds with free radical scavenging activity (Yildiz & Eyduran, 2009) and/or increasing milk protein polyphenol interaction (Yuksel, Avci, & Erdem, 2010).

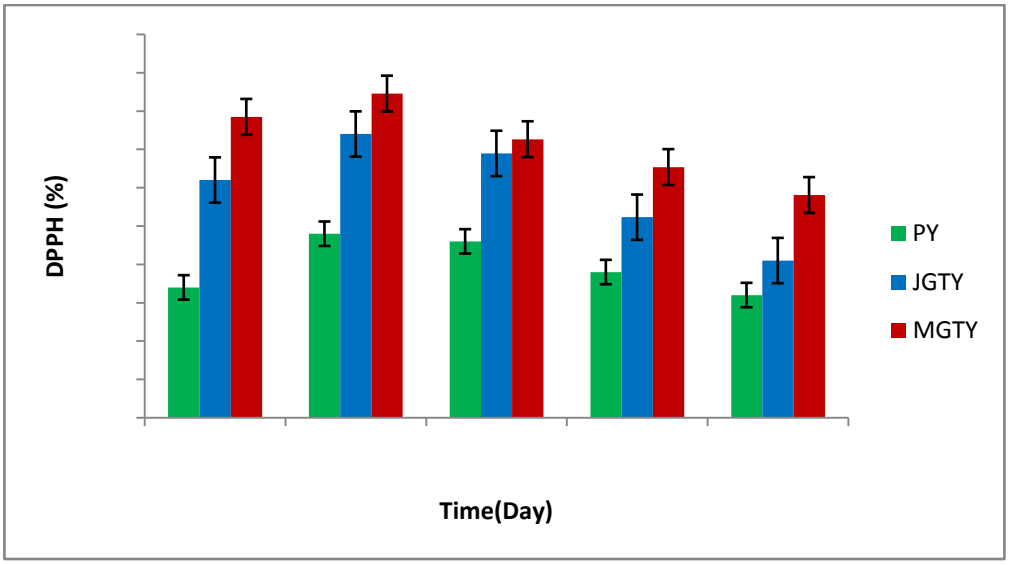

Figure 6.5 Free radical scavenging activity (% inhibition of DPPH oxidation) by plain- and green tea yogurts was determined during 28 day of refrigerated (4 ˚C) storage.
MGTY =Malaysian green tea yogurt, JGTY= Japanese green tea yogurt. Data are presented as means ± SD (n=3). The antioxidant activity in fresh milk was 8.11± 0.23.

6.2.7 Effect of green tea on ferric reducing antioxidant power (FRAP) of yogurts during storage

FRAP assay, commonly used for the routine analysis of single antioxidants and total antioxidant activity of plant extracts, measures the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) in the presence of antioxidants. The addition of green tea increased FRAP

values with MGTY (14.19± 3.67 mmol /L) being higher than JGTY (3.79 ± 1.06 mmol /L) compared to PY (1.25± 0.45 mmol /L). The increase in the FRAP values of tea-yogurts is in line with earlier studies (Najgebauer-Lejko et al., 2011) which showed the ability of ferric ion reduction in tea yogurts being 2-12 times higher, depending on the type of tea and the level of supplementation. The reduction of FRAP values in samples with storage implies further degradation of these products possibly by residual microbial activities (Chen, Lindmark-Mansson, Gorton, & Akesson, 2003, Figure 6.6).

The principle function of antioxidants from food preservation point of view is in delaying the oxidation of nutrients by inhibiting the initiation or propagation of oxidizing chain reactions caused by free radicals. This is advantageous for preserving food quality because the development of rancidity or other off-flavor attributes due to lipid oxidation (Oktay, Gülçin, & Küfreviolu, 2003) can be delayed or even prevented. Extracts from natural antioxidant such as green tea have stronger antioxidant activities than those of synthetic antioxidants. Thus the addition of green tea can increase the antioxidant contents and the stability of yogurt during storage.

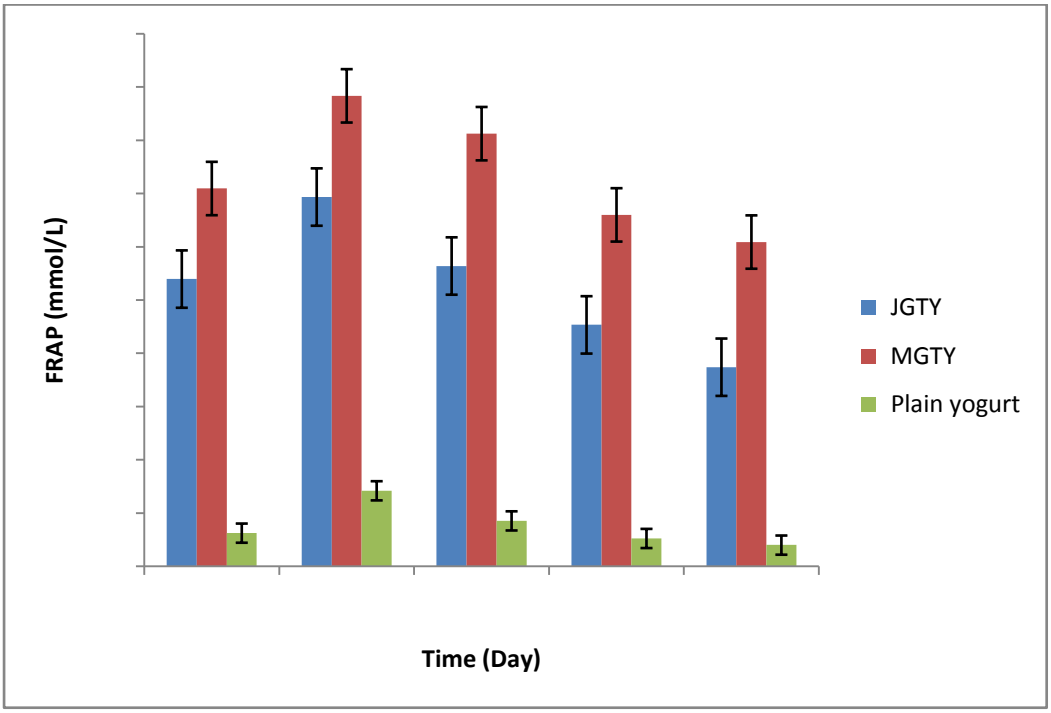

Figure 6.6 Ferric reducing antioxidant power (FRAP; mmol /L) by plain- and green tea-yogurts were determined during 28 day of refrigerated (4 ˚C) storage. MGTY =Malaysian green tea yogurt, JGTY= Japanese green tea yogurt. Values are means ±SD (n=3)

6.2.8 Effects of green tea on inhibition enzymes by yogurts during storage

5.3.8.a Alpha-amylase inhibition

The inhibition of α-amylase activity by yogurt extracts is as shown in Table 6.3. Plain yogurt on day 1 of storage inhibited 10.91% of α-amylase activity. The inhibition of α-amylase increased to 12.32% by day 7 of storage followed by decreased gradually to the lowest (8.55%) on day 28 of storage.

MGT and JGT-yogurts had higher α-amylase inhibition (25.49 and 26.63% respectively) on day 1 of storage compared with plain yogurt (10.91 %). Both green tea-yogurts showed higher ($p<0.05$) α-amylase inhibition on day 7 (41.4 and 40.0% for MGTY and JGTY respectively) compared to that shown for plain yogurt (12.32%). However the

rates of reduction of inhibition on α-amylase was lower in JGTY (7.77 %) followed by MGTY (15.17%) and PY (27.6 %) during day 7 to day 28 of storage.

Table 6.3 Changes in percent (%) α-amylase inhibition of plain and green tea –yogurts during storage at 4˚C

Storage day	1	7	14	21	28
MGTY	25.49±2.32[a]	41.4±2.17[a]	38.82±0.98[a]	31.16±1.09[a]	22.06±1.21[a]
GJTY	26.63±1.42[a]	40.0±1.65[b]	41.56±2.50[a]	32.11±1.80[a]	24.71±1.56[b]
PY	10.91±1.25[b]	12.32±2.80[a]	11.47±1.90[b]	10.04±2.20[a]	8.55±1.50[b]

MGTY=Malaysian green tea yogurt, JGTY=Japanese green tea yogurt, PY= plain yogurt. Results are presented as mean± SD of three observation (n=3).
[a, b] Different superscript within the same row of each sample differ significantly ($p<0.05$)

Amylase is present in both salivary and pancreatic secretion (Ramasubbu et al., 2004) and is responsible for cleaving large malto-oligosaccharides to maltose, a substrate for intestinal α-glucosidase (Loizzo, Saab, & Tundis, 2008).

The ability of the alpha-amylase enzyme inhibitors to avoid dietary starch to be digested and absorbed in the organism has allowed to designateing these compounds as starch blockers. However, only a mild pancreatic alpha-amylase inhibition activity is recommended in order to prevent the abnormal bacterial fermentation of undigested carbohydrates in the colon as a result of an excessive inhibition of this enzyme, which results in flatulence and diarrhea (Tundis, Loizzo, Menichini, 2010).

MGTY and JGTY showed more pronounced inhibition on α-amylase activity than plain yogurt (Table 6.3).The potency of α-amylase inhibition by yogurts (IC50 value) decreased in first 7 day of storage. Plain yogurt showed higher IC50 value than green tea

yogurts (Fig 6.7). The consumption of yogurt has been suggested to be beneficial to those with diabetes (Salminen, Gueimonde, & Isolauri, 2005) and this is attributed partly to the anti-α-amylase activities. The present studies showed that the therapeutic benefits gain from yogurt (Chawdhury, Chakraborty, & Raychaudhuri, 2008) may be further enhanced by the addition of certain green tea extracts. Many secondary chemical compounds synthesized by plants are associated with plant defence. These include enzyme inhibitors (Schuler, Poppy, Kerry, & Denholm, 1998) which impede digestion through their action on insect gut digestive a-amylases and proteinases, hence the digestion of plant starch and proteins (Ryan, 1990). In fact the use of many herbal medicines the prevention and treatment of diabetes depended mostly on the phytochemicals regulation of intestinal enzyme activities. The total phenolic content in green tea yogurts showed good correlation with α-amylase inhibition, with MGTY being stronger than JGTY (MGTY r^2 = 0.93 and JGTY r^2 = 0.84 respectively).

Phytochemicals may consist of hundreds of compound which can be may categorized as natural monophenols, flavonoids, phenolic acids, hydroxycinnamic acids.The enhanced anti-α-amylase activities of the green tea-yogurts during refrigerated storage, at least during the first 7 days of refrigerated storage. Points out the possibilities is these phytochemicals may be converted to more active forms during storage. Phenolic compounds play important roles in protein precipitation and subsequently enzyme inhibition, through forming various complexes (Shi, He, & Haslam, 1994; He, Shi, & Yao, 2006). In particular the hydroxyl and galloyl groups in the molecular structure of tea polyphenols such as tannic acid, gallotannin, catechin and proanthocyanidin can react with proteins through non-covalent interactions (Dreosti, 2000) with the polar groups (amide, guanidine, peptide, amino and carboxyl groups) of protein resulting in the changes of

enzyme molecular configuration and lead to the loss of catalytic activity (Kennedy, Kandel, Cross, & Hay, 1999).

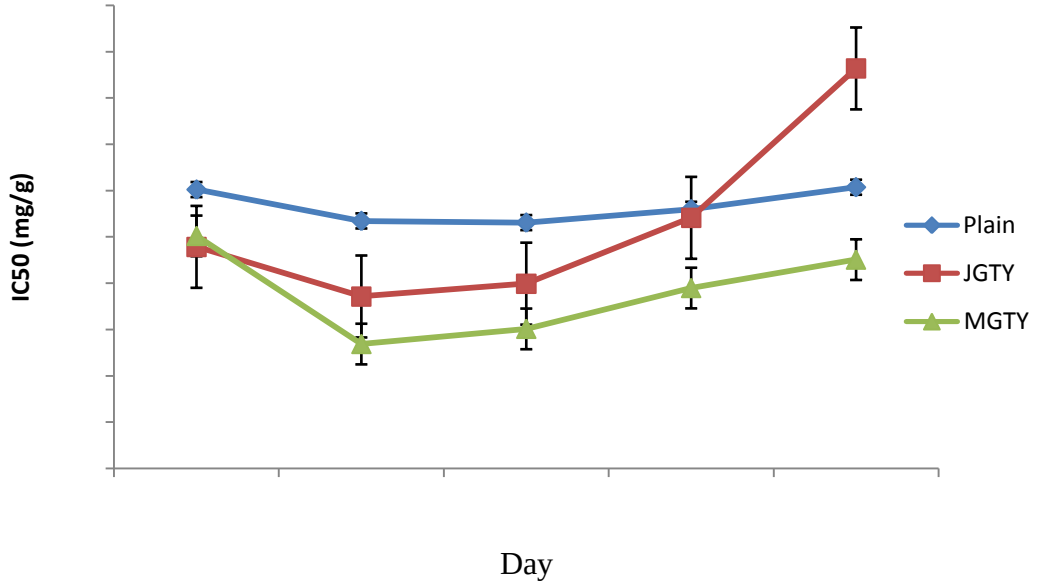

Figure 6.7 IC_{50} value for α-amylase inhibition by plain and green tea-yogurts during storage at 4˚C. Data are presented as mean± SD, (n=3)

5.3.8.b Alpha- glucosidase inhibition

The control of postprandial hyperglycemia is critical in the early therapy for diabetes. One therapeutic approach to decrease postprandial hyperglycemia is to retard the absorption of glucose by inhibiting enzymes such as α-glucosidase (Kim, Jeong, Wang, Lee, & Rhee, 2005). The ability of the green teas and plain- yogurts to inhibit the activity of α- glucosidase is shown in Table 6.4. All yogurts showed maximal inhibition on α-glucosidase on day 7 and 14 of storage followed by gradual reduction in inhibitory activity thereafter.Both green tea yogurts showed higher inhibition than plain yogurt on day 1 of

storage (22.3, 20.5 and 11% for JGT, MGTY and PY respectively). The potency of α-glucosidase inhibition by the three yogurts (IC_{50} values; Fig. 6.8) decreased in first week of storage at 4°C and remained unchanged ($p>0.05$) until the 14th day followed by a gradual increase towards the end of the storage period (day 28). Plain yogurt showed higher IC_{50} value ($p<0.05$) on α-glucosidase inhibition over the storage period. Other yogurt samples produced lower IC_{50} values of α-glucosidase in the presence of green tea (Figure 6. 8).

α-glucosidase is an enzyme located at brush border of microvillus in small intestine. This enzyme is a regulatory enzyme where it catalyzes the final step of carbohydrate metabolism i.e. oligo- and di-saccharides to monosaccharides prior to absorption into the blood circulation (Kawabata et al., 2007). Both green tea yogurts showed maximal inhibition on α-glucosidase on day 7 and 14 of storage (Table 6.4) followed by gradual reduction inhibitory activity thereafter. The profound effects of these plants may be attributed to flavonoids and polyphenols, as well as polimerized form of phenols/ phenolics or their sugar derivatives (Yazdanparast & Alavi, 2001). Although the relative content of these compounds are not known from the present studies, they can collectively contribute to effective inhibitions of α-glucosidase (McCue & Shetty, 2005; Kaushik & Hemre, 2008).

Table 6.4 Changes in percent (%) α-glucosidase inhibition of plain and green tea –yogurts during storage at 4˚C

Storage day	**1**	**7**	**14**	**21**	**28**
plain	11.0±1.40[a]	19.8±1.80[a]	24.3± 1.10[a]	20.1± 2.20[a]	16.0±2.50[a]
JGTY	22.3±1.60[a]	29.6± 0.98[b]	32.9± 1.00[a]	25.1±1.30[b]	20.1± 0.96[a]
MGTY	20.5±2.30[a]	27.7±1.20[b]	31.7±1.20[a]	25.6 ±1.50[b]	23.5±2.10[b]

MGTY=Malaysian green tea yogurt, JGTY=Japanese green tea yogurt, PY= plain yogurt. Results are presented as mean± SD of three observation (n=3).

[a, b] Different superscript within the same row of each sample differ significantly ($p<0.05$)

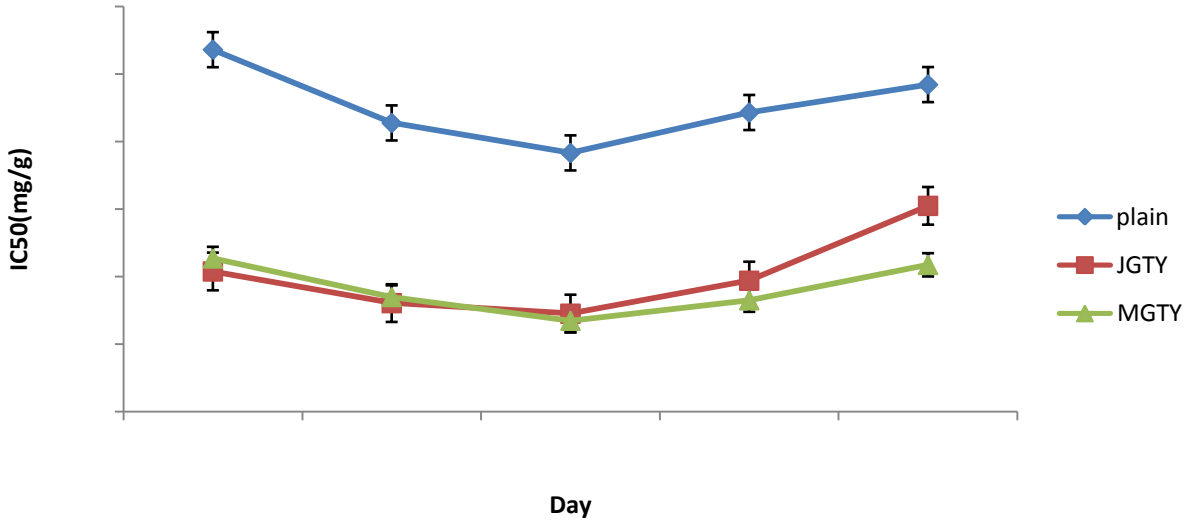

Figure 6.8 IC_{50} value for α- glucosidase inhibition by plain and green tea-yogurts during storage at 4˚C. Data are presented as mean± SD, (n=3)

6.2.9 Changes of green tea polyphenolic componds during refrigerated storage of yogurt as determined by LC/MS

LC-MS method was used to quantify polyphenolic antioxidants in the present study. LC-MS was useful in this study because the liquid chromatograph provided an initial separation of the mixture of compounds based on their polarities. This method allowed the major polyphenolic constituents of the two green tea yogurts to be distinguished and quantified (Ma, Yang, Basile, & Kennely, 2004). This is the 1st report on changes in bioactive green tea compounds during long-term storage of green tea yogurt.

Free radical scavenging capacity and antioxidant power of phenolics has attracted much attention of food and health scientists (Macheix, Fleuriet, & Billot, 1990). An

important feature of these compounds such as flavonoids and anthocyanins is that the antioxidant potentials can even be higher than vitamin-C, vitamin-E and β-carotene (Eberhardt et al., 2000). Thus the stability of the phenolic compounds during storage needs to be ascertained so that the fermented product can be claimed to contain rich sources of antioxidants.Table 6.5 and 6.6 showed MGTY and JGTY respectively contained bioactive compounds during 28 day of storage. Four major compounds were detected in fresh MGTY. In fresh MGTY the highest concentration was shown by quercetin-rhamnosylgalactoside (8.90 mg ml^{-1}) followed by gallocatechin (7.34 mg ml^{-1}), kaempferol -3 –rutinoside (6.41 mg ml^{-1}) and epicatechin (6.43 mg ml^{-1}) (Figure 6.9). During storage (Figure 6.9- 6.13), the content of gallocatechin, the most abundant catechin in the tea varieties, decreased by 10.54%, whereas epicatechin, the second most abundant catechin, decreased by 18.20%. These results indicate that epicatechin may be more susceptible to degradation than gallocatechin. Gallocatechin is reported as being more abundant than epicatechin in most types of tea, perhaps due to it being more stable (Chaturvedula & Prakash, 2011). During refrigerated storage MGTY was shown to have its quercetin-rhamnosylgalactoside, decreased by 55.32%, whereas kaempferol -3–rutinoside, decreased by 29.23%. The result suggests that anthocyanidin are more stable than flavanoids in MGTY during 28 day of storage (Figure 6.3).

The concentration of four major bioactive compounds in JGTY (Figure 6.14) also showed gallocatechin (5.81 mg mL^{-1}) being the highest in fresh yogurt followed by quercetin-3-O-galactosyl-rhamnosyl-glucoside (5.92 mg ml^{-1}), epicatechin (4.96 mg mL^{-1}) and quinic acid conjugate (1.58 mg mL^{-1}). Refrigerated storage increased ($p< 0.05$) the value of phenolic compounds on the 7th day of storage (Figure 6.15) for epicatechin and quercetin-3-O-galactosyl- rhamnosyl-glucoside (6.71 and 9.21 mg mL^{-1} respectively)

followed by gradual decrease for all compounds till 28 day of storage (Figure 618). Other compounds were unchanged on day 7 of storage. The rate of decreased was higher in quinic acid conjugate (75%), followed by, quercetin-3-O-galactosyl-rhamnosyl-glucoside (55%),epicatechin (25.25%)and gallocatechin (23%).The anthocyanidins appeared to be more stable than flavanoids in JGTY since epicatechin and gallocatechin changed little during the 28 days storage, which reflects the stability of anthocyanins in pH ranging 4-6 (Cabrita, Fossen, & Andersen, 2000). Plain yogurt did not show any of the phenolic compounds those observed in green tea yogurt extracts, thus may be explain by low antioxidant activity of this type of yogurt.

Both tea yogurts showed the same continuous decrease in value of phenolic compounds with time. One possibility is the oxidation of phenolic by polyphenol oxidase (PPO) (Altunkaya & Gokmen, 2008) which was also reported by Piretti, Gallerani, & Pratella, (1994) and Shiri, Ghasemnezhad, Bakhshi, & Dadi, (2011) whereby catechin, epicatechin and quercetin glycosides decreased during storage. Flavonoids are phenolic derivatives present in substantial amounts in green tea. The decrease in flavonoid is associated with antioxidant capacity during storage. The flavonoids such as quercetin and catechin are common PPO substrates (Nagai & Suzuki, 2001) and both can be oxidized directly by PPO (Jiménez & García-Carmona, 1999). Therefore, the decrease of phenolic composition could be due to the oxidation by PPO (Yamaguchi et al., 2003). The second possible source of variability in anthocyanidin and flavanoids content could be due to genetic variability among the plants from which the leaves were harvested and/or to soil composition, climate, harvesting practices, postharvest storage, sampling, and manufacturing practices (Rusak, Komes, Likić, Horžić, & Kovač, 2008); Sultana, Anwar,

& Iqbal, 2008). Different tea varieties are harvested in different ways and at different times of a year, so that the plants are subjected to different environmental stress conditions.

The major tea compounds characterized in green tea in the present studies have previously been described with respect to health benefits associated with the habits of daily tea consumption (Chacko, Thambi, Kuttan, & Nishigaki, 2010; Chaturvedula & Prakash, 2011). Quercetin-rhamnosylgalactoside and quercetin-3-O-galactosyl-rhamnosyl-glucoside showed the highest range of value in MGTY and JGTY which contribute as the main constituents of antioxidant power of tea-yogurts. The presence of quercetin, a major representative of the flavonol subclass in MGT and JGT-yogurt, contributes importantly to the color of green tea (Yang, Wang, Lu, & Picinich, 2009). This flavonoid is known effective in preventing the oxidation of LDL by scavenging free radicals and chelating transition metal ions (Yao et al., 2012) by virtue of the dihydroxylated B-ring and unsaturated C-ring. As a result, quercetin may aid in the prevention of certain diseases, such as cancer, atherosclerosis and chronic inflammation (Catea, Francisco, Soengas, & Velasco, 2010). The pronounced antioxidant activities of kaempferol also was associated with the prevention of arteriosclerosis by inhibiting the oxidation of low density lipoprotein and the formation of platelets in the blood (Tzeng et al., 2011; Park, Rho, Kim, & Chang, 2006) apart from reduced incidence of cancer formation (Lopez-Sanchez, Esteban, & Garcia-Rojas, 2007).

Other tea compounds may also have similar actions. Epicatechin which is also a strong antioxidant, reduces lipid peroxidation, inhibits platelet aggregation, has insulin mimic action and improves heart health (Othman et al., 2010). Epicatechin may also cause blood vessel to dilate by regulating formation of nitric oxide, a molecule secreted by the blood vessel endothelium to signal surrounding muscle to relax.

Other green tea compounds also have interesting health attributes although the mechanisms of actions are not yet fully established. Gallocatechin for instance may be effective in preventing hyperlipidemia by lowering plasma and hepatic cholesterol concentrations (Ishizu, Tsutsumi, & Sato, 2009).

The concentration of major compounds in green tea yogurts was reported are more than plain yogurt but less than green tea infusion which is in agreement with the negative effects of milk to tea on its antioxidant properties (Sharma, Vijay Kumar, & Jagan Mohan Rao, 2008). However, the reduction in green tea compounds, thus antioxidant activities, when added into milk may actually due to quantitative losses as a result of extraction (Dubeau, Samson, & Tajmir-Riahi, 2010). This is because the addition of milk to tea did not significantly affect the blood catechin level (Van het Hof, Kivits, Weststrate, & Tijburg, 1998). Hence, it should be borne in mind that the antioxidant capacity of yogurts supplemented with green tea infusions greatly depended both on the type of tea used (type of polyphenolic compounds) as well as its concentration (the amount of polyphenols and milk protein: tea polyphenols ratio).

In solution, polyphenols such as catechins can form insoluble complexes (Liang, Lu, Zhang, Wu, & Wu, 2003), by interacting with proline-rich proteins such as β-casein, the most abundant milk protein. The binding affinity of polyphenols to proteins is dependent on their molecular size (De Freitas & Mateus, 2001). Larger polyphenols like those present in green tea (theaflavin, thearubigin) due to the fermentative oxidation/polymerisation of catechin monomers, are therefore more likely to bind milk proteins (Art et al., 2002). This binding can affect catechin antioxidant activity by lowering the number of free hydroxyl groups (Heim, Tagliaferro, & Bobilya, 2002). Although no relevant effect of milk on the bioavailability or on the antioxidant capacity of tea

polyphenols were observed in other studies (Leenen, Roodenburg, Tijburg, & Wiseman, 2000); Van het Hof et al., 1998). Schroeter, Holt, Orozco, Schmitz, & Keen, (2003) observed that the presence of milk in cocoa products also did not counteract absorption and biological activity of flavonol monomers, nor did it affect plasma antioxidant capacity. Polyphenols can react with the amino groups of peptides, leading to the formation of protein cross-links (Van het Hof et al., 1998). Ferulic acid for instance is an abundant phenolic phytochemical found in plant cell wall and it can cross-link with protein and polysaccharides by donating hydrogen from its phenolic hydroxyl group and producing a resonance-stabilized free radical intermediate (Hollman, Van het Hof, Tijburg, & Katan, 2001).

Most phenolic compounds have high reactivity and thus can be easily affected by changes in pH, extreme temperature, enzymes, microbial activity, acids and metallic ions present in the environment (Karaaslan et al., 2011).These result in degradation and thus explains the substantial decrease in phenolic compounds foods such as virgin olive oil and broccolis during storage (Lemoine, Civello, Martinez, & Chaves, 2007). Similar observation was seen in the decrease in TPC (Table 6.2) which readily equates to reduced antioxidant capacity (Table 6.3 and 6.4) of green tea yogurts. However bearing in mind that the amount of TPC present in green tea yogurts are exceptionally high, the strong antioxidant capacity of epicatechin in green tea (Lee, Kim, & Lee, 2003) to scavenge free radicals present in the body (Choo, 2003) may be benefited by consumers even after 2-3 weeks of refrigerated storage. The present studies provide further evidence that the inclusion of tea in yogurt can increase food functional values by virtue of tea polyphenolic compounds.

Table 6.5 Concentration of bioactive compounds (mg mL^{-1}) in Malaysian green tea yogurt extract during 28 days of refrigerated storage

Phenolic compounds	**Day1**	**Day 7**	**Day 14**	**Day 21**	**Day28**
Quinic Acid Conjugate	1.15	ND	ND	ND	ND
Gallocatechin	7.34	5.14	5.21	1.15	1.09
Chlorogenic Acid	0.003	ND	ND	ND	ND
Epicatechin	5.43	5.52	6.61	6.05	5.44
6-C-glucosyl-8-C-arabinosyl apigenin	0.034	ND	0.001	0.001	0.001
Myricitin-3-O-glucoside	0.003	ND	ND	ND	ND
Quercetin-rhamnosylgalactoside	8.90	12.28	9.63	8.79	5.73
Kaempferol 3 rutinoside	6.41	1.57	1.21	1.00	4.96
Kaempferol-3-O-glucoside	0.003	ND	ND	ND	ND
Quercetin-3-O-galactosyl-rhamnosyl-glucoside	ND	0.22	0.04	ND	ND

ND: Not Detected

Table 6.6 Concentration of bioactive compounds(mg mL^{-1}) in Japanese green tea yogurt extract during 28 days of refrigerated storage

Phenolic compounds	**Day 1**	**Day 7**	**Day 14**	**Day 21**	**Day28**
Quinic Acid Conjugate	1.58	1.06	1.78	1.01	0.9
Gallocatechin	5.81	ND	5.30	4.96	4.74
Epicatechin	4.96	6.71	5.86	3.83	3.96
Dicaffeoquinic Acid Conjugate	0.17	0.18	ND	ND	ND
6-C-glucosyl-8-C-arabinosyl apigenin	0.75	1.11	0.20	0.31	0.28
quercetin-rhamnosylgalactoside	1.30	ND	ND	ND	ND
Kaempferol 3 rutinoside	0.29	0.26	ND	ND	ND
quercetin-3-O-galactosyl-rhamnosyl-glucoside	5.92	9.21	6.60	4.00	3.82

ND: Not Detected

6.3 Conclusions

The antioxidant capacity of yogurt can be increased by the addition of green tea. Total phenolic contents and the antioxidant properties were highly correlated (R^2= 0.8, 0.6 and 0.87 for MGTY, JGTY and PY respectively) which implies yogurt antioxidants with or without green tea are potent scavengers of free radicals in aqueous systems as reported for other foods and beverages.The addition of green tea in the fermented milk used for making yogurts is recommended because green tea is a natural herbal product with a wide range of beneficial and nutritional properties. This green tea yogurt can be regarded as a new yogurt with improved functional properties because of the increased polyphenolic bioavailability. Storage time increased significantly ($p<0.05$) the value of catechins content in yogurt but this depends entirely on the type of green tea used in the fermentation of milk. In general the consumption of green tea yogurt is recommended within 14 days of refrigerated storage to maximize the best nutritional value and health benefits.

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