CHAPTER V

EVALUATION OF ANTIOXIDANT ACTIVITY AND MICROBIAL GROWTH IN GREEN TEA YOGURT

5.1 Introduction

Fermented milk products have numerous health benefits due to the functional properties of their viable microorganism such that a health claim for yogurt is allowed towards improved lactose digestion for individuals with lactose mal-digestion (EFSA Panel on Dietetic Products, 2010) . In addition, milk fermented by selected culture of lactic acid bacteria (LAB) can have high biochemical and antioxidant activities (Kullisaar et al., 2003; Villani et al., 2005). The addition of plant materials, particularly fruits, into yogurt is mostly practiced to increase the appealing sour taste of yogurt (Clark & Plotka, 2004). Non-conventional approach of adding aromatic plants, fibre etc., have also been attempted to improve physio-chemical and nutritional properties of yogurt. In the case of green tea, its addition into yogurt can impart unique enhancing properties associated with tea flavor and antioxidant properties and health-promoting benefits by virtue of phenolic compounds. The

tea polyphenolics, particularly catechins, are the responsible antioxidant compounds used in many foods such as meats, fishes and vegetable oils (Yilmaz, 2006). Thus the addition of green tea to milk before fermentation would improve the growth and stability of yogurt bacteria during fermentation and subsequently increased yogurt nutritional properties.

Reactive oxygen species (ROS) and free radicals play important roles in many degenerative diseases like cancer, atherosclerosis and diabetes (Beckman & Ames, 1998). Formation of free radicals, such as superoxide anion radical and hydroxyl radical, is an unavoidable consequence in aerobic organisms during respiration. These radicals are very unstable and react rapidly with other groups or substances in body, leading to cell or tissue injury (Hamid, Aiyelaagbe, Usman, Ameen, & Lamal, 2010). The body has its own defence system against ROS based on antioxidant enzymes, as well as low molecular mass non-enzymatic antioxidant compounds. These defence systems are not effective enough to totally prevent the damage, and therefore, food supplements containing antioxidants may be used to help the human body to reduce oxidative damage (Terahara, Kurama, & Takemoto, 2001; Kullisaar et al., 2003) and subsequently the risks of getting the degenerative diseases.

The main objectives of this study were 1) to determine the effects of green tea on the formation of organic acids, crucial in the acidification process of yogurt, during the fermentation of milk; 2) to investigate effects of green tea on antioxidant activity of yogurt during fermentation; 3) to establish the effects of green tea on the growth of *Lactobacillus spp.* and *S. thermophillus*.

5.2 Materials and Methods

Milk, either neat or infused with green tea (see Section 3.1), was inoculated with

yogurt bacteria (see Section 3.3) and subjected to incubation at 41˚C until the pH of milk fermented reached 4.5. Samples of fermented milk were taken during every hour of the incubation and these were directly used in the analysis of microbial growth and measurement of physio-chemical properties. Non-hydrolysed casein was removed from the fermented milk prior to the antioxidant activity measurement. Briefly, the pH of fermented milk (15 ml) was adjusted to 4.6 by adding 1M HCl. The suspension was centrifuged (5000g, 20 min, 5˚C) and the supernatant was filtered through a 0.45-µm filter. The filtrate was stored at -20˚C for further analysis. For organic acid analysis the samples taken at each hour of the fermentation were centrifuged at (5000g, 10 min, 4 °C) and the supernatant directly used in the preparation for LC-MS analysis.

5.2.1 pH and titratable acid (TA) determination

The pH and TA of all samples were measured as indicated in Section 3.7.

5.2.2 Analysis of total phenolic content (TPC)

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

5.2.3 Detemination of antioxidant activity

The DPPH inhibitory activities as well as FRAP values of yogurt were determined according to procedure described in Section 3.9.1 and 3.9.2 respectively.

5.2.4 Determination of viability of lactic acid bacteria (LAB)

Viable LAB in yogurt was enumerated as described in Section 3.15.

5.2.5 Determination of organic acids

The method of extraction of samples for determination of organic acids was described in Section 3.16.1 and measurement of concentration of organic acids in all samples was described in Section 3.17.

5.2.6 Proteolytic activity by O-Phthalaldehyde (OPA) method

Proteolytic activity of all samples was determined as described in Section 3.10.

5.3 Results and Discussion

5.3.1 Effects of green tea on acidification during the fermentation of milk

pH of yogurts was measured during fermentation until pH 4.5 unit/h was obtained (Table 5.1). The addition of green tea water infusion did not affect the pH of milk-starter culture at the beginning of the incubation (ranged 6.31- 6.38 pH unit/h) indicating the acidic nature of the green tea, if any, were minimal. However the difference in reduction of pH between green tea- and plain-yogurts became significant during 60-180 minutes of incubation. Faster reduction of pH was observed for MGT and JGT (-0.40 pH unit/h and -0.42 pH unit/h respectively) than for PY (-0.30 pH unit/h). As a result, the samples fortified with green tea reached a pH of 4.5 significantly earlier (300min) than plain yogurt (360min; $p<0.05$). Decreased fermentation time was also reported for the fermentation of milk in the presence of lentil flour (Zare, Champagne, Simpson, Orsat, & Boye, 2012). The greater acidification rates in green tea yogurts could be partially due enhanced growth of lactic acid bacteria (Tamime & Robinson, 1999) stimulated by the presence of green tea. The reduction time for fermentation of milk implicate positive attribute of green tea with respect to energy savings in the manufacturing of yogurt.

Titratable acid (TA) measures acids that can lose proton(s) in an acid-base reaction and it reflects total acid regardless of strong (readily dissociable H+) or weak (organic acids) acids (Kurman & Rasic, 1991). In the present studies TA represents the amount of acidic compounds formed as a result of yogurt bacteria catabolism of milk lactose (Afonso & Maia, 1999; Beal, Skokanowa, Latrille, Martin, & Corrieu, 1999) and is conveniently used in the evaluation of fermentation capacity of microbes (Geidam, Ambali, & Onyelili, 2007). The presence of green tea infusion did not affect TA of milk-starter culture mixture at the beginning of fermentation. However the rates of acid formation in green tea yogurts were higher than that for plain-yogurt during fermentation (Table 5.2). MGT yogurt showed faster rate of acid formation followed by JGT and plain-yogurt (2.29, 1.96 and 1.7 unit/h respectively) The rate of fermentation is affected by many parameters including the milk base, the type and concentration of supplemented ingredients, starter culture, incubation temperature, and milk heat treatment (Soukoulis, Panagiotidis, Koureli, & Tzia, 2007). The determination of TA is thus relevant in the evaluation of fermentation capacity of microbes (Geidam et al., 2007). In fact organic acids produced in yogurt (lactic acid, citric acid, formic acid, acetic acid and butyric acid (H. M. Ostlie, Treimo, & Narvhus, 2003) are linearly related with the accumulation of TA (Billard, Mekki, Ouadi, & Gaillard, 2007). Variations in titratable acidity that occur in yogurts in the present study can thus be associated to differential microbial population during fermentation (Eissa, Mohamed Ahmed, Yagoub, & Babiker, 2010).

Table 5.1 Changes of pH during fermentation of milk with green tea at 41˚C

Time (min)	Plain-Y	MGT-Y	JGT-Y
0	6.38±0.05[a]	6.31±0.07[a]	6.35±0.06[a]
60	6.21±0.09[a]	6.22±0.08[a]	6.18±0.10[a]
120	5.91±0.02[a]	5.67±0.06[a]	5.63±0.16[a]
180	5.43±0.10[b]	5.25±0.08[a]	5.15±0.15[b]
240	5.17±0.08[b]	4.97±0.27[b]	4.77±0.20[b]
300	4.72±0.23[b]	4.49±0.09[b]	4.51±0.14[b]
360	4.50±0.11[b]	-	-

Milk was fermented by starter culture in the absence Plain (PY) or presence of 2% w/v of Malaysian (MGT-Y) or Japanese (JGT-Y) green tea.(-): means stop measuring pH. Values are presented as means ± SD (n = 3). Different superscript [a,b] in the same row differ significantly at $p<0.05$

Table 5.2 Changes of titratable acid (TA; % lactic acid equivalent) of green tea yogurts during fermentation of milk at 41˚C

Time (min)	Plain-Y	MGT-Y	JGT-Y
0	0.30±0.03[a]	0.27±0.02[a]	0.30±0.01[a]
60	0.36±0.02[a]	0.36±0.03[a]	0.42±0.02[a]
120	0.51±0.09[b]	0.48±0.03[a]	0.57±0.07[b]
180	0.58±0.11[b]	0.63±0.05[b]	0.78±0.10[b]
240	0.66±0.07[b]	0.72±0.06[b]	0.86±0.07[b]
300	0.79±0.11[b]	0.89±0.05[b]	0.89±0.07[b]
360	0.81±0.10[b]	-	-

Milk was fermented by starter culture in the absence Plain (PY) or presence of 2% w/v of Malaysian (MGT-Y) or Japanese (JGT-Y) green tea. Values are presented as means ± SD (n = 3). Different superscript [a,b] in the same row differ significantly at $p<0.05$

5.3.2 Effects of green tea on total phenolic content (TPC) of fermented milk

As shown in Figure 5.1 The addition of green tea into milk increased TPC content of milk (4.8 μgGAE/mL) at the first hour of fermentation to 18.4 and 28.9 μgGAE/mL for JGT- and MGT-milk mixtures respectively. The TPC content at the end of fermentation increased to 7.32, 25.03 and 35.7μgGAE/mL for PY, JGTY and MGTY respectively. Increased TPC values in PY compared to milk represent phenolic compounds released from milk protein breakdown (Damin, Alcântara, Nunes, & Oliveira, 2009). The amino acid tyrosin for instance has a phenolic side chain suggested (Shah, 2000) to give rise to the reading in TPC. Another possibility is that microbial utilization of phenolic acids such as ferulic and p-coumaric acid during fermentation process and post acidification lead to the production of other phenolic acids such as vanillic and p-hydroxybenzoic acids before the aromatic ring structure is broken down (Blum, 1998). Since TPC measures phenolic compounds in milk and green tea extracts, it is reasonable to assume that the increase in TPC values in tea yogurts were contributed by further breakdown of phenolic compounds from both milk and green tea during fermentation as a result of microbial metabolic activities. For instance, during fermentation, enzymes such as amylases and proteases derived from the peptides or microbes contribute to the changes in yogurt (Katina, Liukkonen, Kaukovirta-Norja, & Adlercreutz, 2007; Loponen, Mikola, Katina, Sontag-Strohm, & Salovaara, 2004).

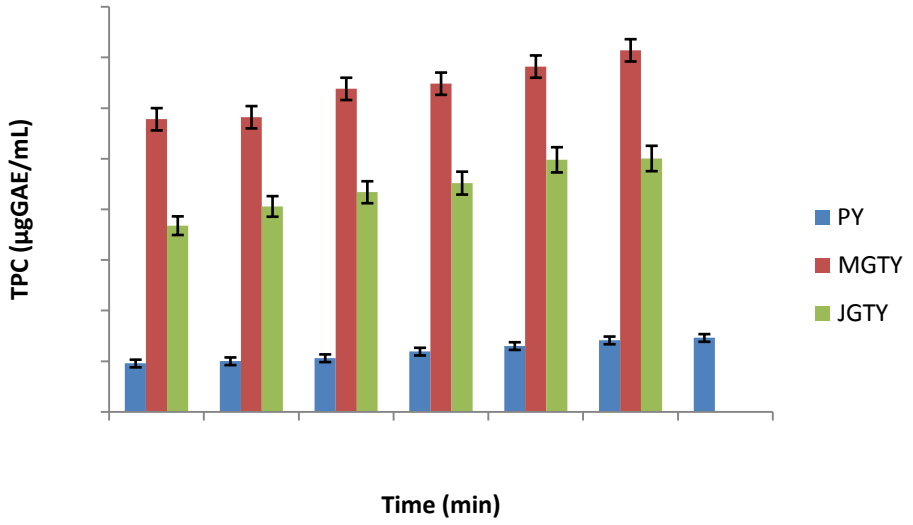

Figure 5.1 Total phenolic concentrations (µgGAE/mL) in plain- and green tea-yogurts during fermentation. MGTY=Malaysian green tea yogurt, JGTY=Japanese green tea yogurt, PY= plain yogurt. Values are means ± SD (n = 3). Total phenols were expressed as µg gallic acid equivalent (µgGAE)/mL.

5.3.3 Effect of green tea on antioxidant activity of fermented milk

The changes in the free radical scavenging activity during fermentation were monitored using DPPH scavenging assay. Milk contains 13.01±0.40% free radical scavenging activity and this was increased to 31.9±0.06% and 28.3±0.27 % after the addition of MGT and JGT respectively (Figure 5.2). The free radical scavenging activity increased in all three fermented milk during fermentation. Greatest inhibition of DPPH oxidation by the end of fermentation occurred in MGTY (39.0±0.77%) followed by JGTY (31.2±0.14 %) and PY (17.43±0.21%). Microbial growth during fermentation is known to alter some of the phenolic compounds and hence their free radical scavenging activity (Blum, 1998). The present studies provided further evidence that apart from the use of specific LAB, the development of free radical scavenging activity in yogurt can be stimulated by the addition plant materials that enhanced microbial fermentation of milk.

This was demonstrated by MGT in particular (Figure 5.2) which could have stimulated LAB bacteria and subsequently the free radical scavenging activity as reflected by the higher rate of increase in DPPH inhibition of MGTY during the 300 min fermentation period (i.e. 1.45%/hr compared to JGTY (0.58%/hr) and PY (0.71%/hr).

Antioxidative property, especially radical scavenging activity, is very important due to the deleterious role of free radicals. The hydroxyl radicals are extremely reactive free radicals formed in biological systems. They react rapidly with almost every type of molecule in living cells, such as amino acids, phospholipids, DNA, bases, and organic acids. The scavenging of different types of ROS is considered to be one of the main antioxidant mechanisms exhibited by lactic acid bacteria (Namiki, 1990; Kim et al., 2006). In this regard the consumption of yogurt may provide scavengers for these hydroxyl radical by virtue of metabolic compounds produced by bacteria or degradation products of milk proteins (Virtanen, Pihlanto, Akkanen, & Korhonen, 2006). The present studies demonstrated that the addition of green tea may not only directly increase the free radical scavenging activity of yogurt but also could enhance the production of antioxidants from the fermentation of milk.

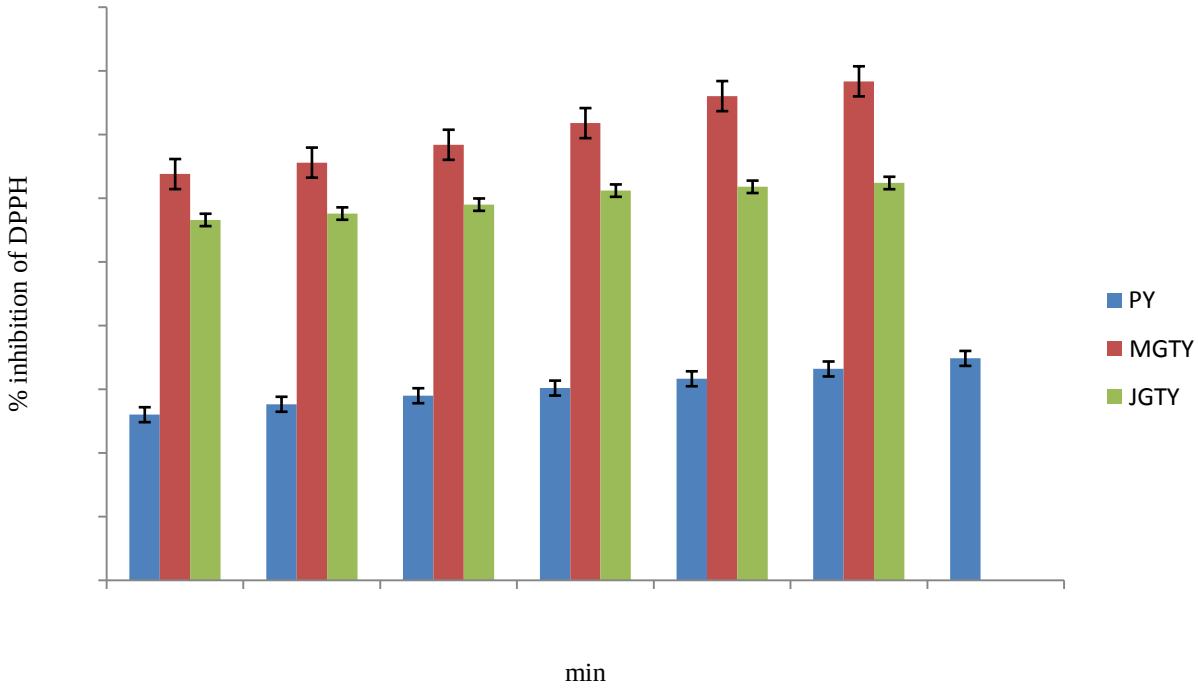

Figure 5.2 Antioxidant capacity (% inhibition of DPPH oxidation) by plain- and green tea-yogurts during fermentation.MGTY=Malaysian green tea yogurt, JGTY=Japanese green tea yogurt, PY= plain yogurt. Values are means ± SD (n = 3)

5.3.4 Effect of green tea on ferric reducing antioxidant power of fermented milk

Ferric reducing antioxidant power (FRAP) of yogurts is shown in Figure 5.3. Fermentation of milk by yogurt bacteria did not result in significant effect on FRAP value and addition of green teas did enhance the ferric reduction power of the yogurts.. This is demonstrated in a small increase in FRAP expressed in mmol /L before (value ie at t=0 min) and after fermentation (value ie at t=300min). Hence most of the increase in FRAP value of yogurt was contributed by either MGT or JGT. The highest FRAP value, was found in MGT-yogurt (12.8 mmol /L), followed by JGT- and plain-yogurts (9.93 and 0.97 mmol /L, respectively) at the first hour of incubation at 41˚C. In contrast to DPPH, both

teas did not cause additional increase in FRAP value during fermentation. There are limited studies on the effects of fermentation influences on FRAP value in yogurt.

However, the results presented here are similar to those reported by Hubert, Berger, Nepveu, Paul, & Daydé (2008) which showed that the initial FRAP power was maintained for up to 6 h of incubation period, with significant decrease occurring when incubation was extended until 48 h.

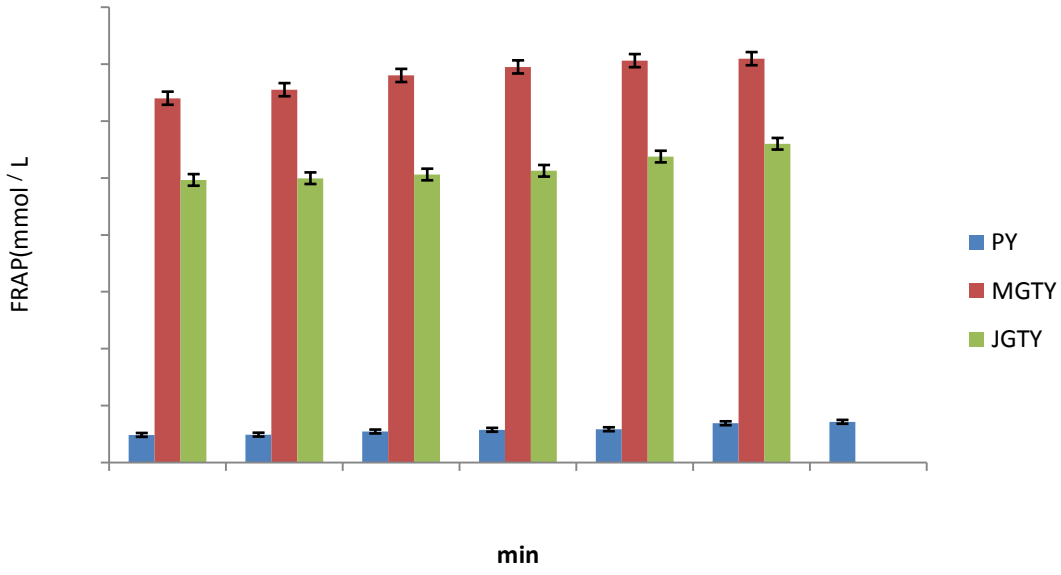

Figure 5.3 The FRAP (ferric reducing antioxidant power) values of yogurts in the absence or presence of green tea extracts. MGTY=Malaysian green tea yogurt, JGTY=Japanese green tea yogurt, PY= plain yogurt. Values are means ± SD (n = 3)

5.3.5 Effects of green tea on yogurt bacteria growth during fermentation of milk

Enumeration of yogurt bacteria was carried out by plating diluted cell suspension on a suitable medium which allowed a viable unit to grow and form a colony. The viable cell counts (VCC) of *Lactobacillus spp.* in inoculated milk increased from 4.6 x 10^8 cfu mL^{-1} at the beginning of incubation to 6.17 x 10^8 cfu mL^{-1} ($p>0.05$) by the end of the fermentation

(Table 5.3). Adding MGT or JGT increased VCC of *Lactobacillus spp* to about 2 fold higher than in plain yogurt by the end of the fermentation. The inclusion of MGT to milk increased (12.54 ±0.70 $x10^8$ cfu mL^{-1} $p<0.05$) bacterial growth higher than the inclusion of JGT to milk (11.06± 0.07$x10^8$ cfu mL^{-1}) at the end of fermentation. Table 5.4 showed the viable cell count of *S. thermophilus* in green tea yogurts. Inoculated milk contained similar number of *S. thermophilus* VCC (77.60±0.75 $x10^6$ cfu mL^{-1}) to those containing green tea (78.03±0.74 and 77.21±1.64 $x10^6$ cfu mL^{-1} respectively for MGT, JGT) at the beginning of incubation. Plain-yogurt had small increase in *S. thermophilus* (83.52±0.27 $x10^6$ cfu mL^{-1}) in comparison to those in the presence of green tea (an increase of 35-50%) by the end of fermentation. There could be substantial differences in MGT and JGT, possibly as a result of differences in the methods of preparation. In particular exposure to extreme heat (steaming process; see section 3.1 which described differences in tea processing for MGT and JGT) in JGT could have resulted in the degradation of compounds and thus making this tea less stimulating to microbial growth compared to MGT.

Milk lactose is converted to lactic acid during fermentation. Previous studies have reported differences in TA in different yogurts despite the fact that yogurt fermentation was stopped at the same pH 4.5 (Cavallini & Rossi, 2009). Thus it is important to relate TA accumulation with cell counts as a result of the presence of green tea during fermentation of milk (Co kun & Ondul, 2004). Higher microbial growth during the fermentation may explain faster reduction in the fermentation time to pH4.5 for green tea yogurts (300; 300 and 360 min for MGT-, JGT- and plain-yogurts respectively).

Certain additives, because of their beneficial contribution to the sensory, therapeutic, or other properties of dairy foods, are used in the manufacture of milk products (Varga & Szigeti, 1998). Some of these additives may contain spoilage microorganisms which may

shorten the shelf life of final products. Thus the addition of green tea with antimicrobial activity against pathogenic bacteria (Chou, Lin, & Chung, 1999; Jaziri & Hamdi, 2005) may have a beneficial role to suppress these microorganisms. This is possible by the fact that the antimicrobial effect of green tea was shown detrimental towards pathogenic bacteria but not towards LAB (Najgebauer-Lejko, Sady, Grega, & Walczycka, 2011). However, this effect may be absent in highly processed tea since Jaziri, Ben Slama, Mhadhbi, Urdaci, & Hamdi (2009) found no effects on the survival of the starter bacteria in yogurts as a result of adding black tea. Thus the eventual benefits of tea on yogurt bacteria growth must be viewed from the potential differences in post-harvest processing affecting amongst others, the antioxidants capacity.

Table 5.3 Viable cell counts (VCC) of *Lactobacillus ssp.* (cfu $x10^8$ mL^{-1}) in green tea yogurts during fermentation of milk

Time (min)	PY	MGTY	JGTY
0	4.61 ± 0.07^a	4.95 ± 0.67^a	4.71 ± 0.53^a
60	4.69 ± 0.06^a	6.86 ± 1.00^a	5.97 ± 1.17^a
120	4.73 ± 0.04^a	8.19 ± 0.76^b	8.91 ± 0.35^a
180	5.51 ± 0.10^b	10.44 ± 1.43^b	9.97 ± 0.07^b
240	5.82 ± 0.04^b	11.17 ± 1.30^b	11.17 ± 0.23^b
300	5.92 ± 0.06^b	12.54 ± 0.70^b	11.06 ± 0.07^b
360	6.17 ± 0.08^b	-	

Bacteria count in yogurts *Lactobacillus spp*. MGTY=Malaysian green tea yogurt, JGTY=Japanese green tea yogurt, PY= plain yogurt. Values are means ±SD (n = 3). Different superscript ^{a,b} in the same row differ significantly at $p<0.05$

Table 5.4 Viable cell counts (VCC) of *S. thermophilus.* (cfu $x10^6$ mL^{-1}) in green tea yogurts during fermentation of milk

Time (min)	PY	MGTY	JGTY
0	77.21 ± 1.64^a	77.60 ± 0.75^a	78.03 ± 0.74^a
60	78.40 ± 1.35^a	85.59 ± 1.22^a	83.53 ± 1.11^a
120	79.31 ± 0.34^b	93.32 ± 1.20^a	88.04 ± 0.16^a
180	81.21 ± 0.28^b	101.17 ± 1.07^b	96.34 ± 1.36^b
240	81.93 ± 0.41^b	108.12 ± 1.49^b	103.61 ± 0.75^b
300	82.20 ± 0.41^b	120.40 ± 1.03^b	118.04 ± 0.17^b
360	83.52 ± 0.27^b	-	-

Bacteria counts in yogurt *S. thermophilus* CFU ($x10^6$ cells/ml), MGTY=Malaysian green tea yogurt, JGTY=Japanese green tea yogurt, PY= plain yogurt. Values are means ±SD (n = 3). Different superscript ^{a,b} in the same row differ significantly at $p<0.05$

5.3.6 Effects of green tea on organic acid production during fermentation of milk

Organic acids are produced during the metabolism of fermentable sugars (Erbaş, Kemal Uslu, Ozgun Erbaş, & Certel, 2006) and they play important roles in development of yogurt flavour and eating quality (Califano & Bevilacqua, 2000). In the food industry and households, various organic acids are added to food to impart a sour taste. These organic acids also serve as an antimicrobial food additive to inhibit spoilage and the proliferation of pathogenic organisms and thus act as natural preservatives to enhance the keeping quality of food products (Golden, Buchanan, & Whiting, 1995; Fang, Lai, & Chou, 2013). Since these acids are products of fermentation, the compositions may reflect the relative extent of various metabolic pathways of yogurt bacteria metabolism (Bevilacqua & Califano, 1992; Lues, 2000). The effect of green tea and fermentation time on the Organic acid content of the fermented milks is presented in Table 5.5. Six major organic acids were detected in all samples.

5.3.6.a Citric acid

Citric acid (2-hydroxypropane-l,2,3-tricarboxylic acid) was first isolated and crystallized from lemon juice. A change in citric acid during fermentation in the present studies is as shown in Table 5.5. The initial level of citric acid in milks immediately after inoculation (0 hour) was 337.63, 467.66 and 321.45 mg/L for MGTY, JGTY and PY respectively. A significant ($p<0.05$) decrease in citric acid content was observed in all fermented milks until end of fermentation. The amount of citric acid was reduced by 60.6, 53.15 and 56.42% for MGTY, JGTY and PY respectively to 132.97, 172.23 and 131.11 mg L^{-1} at the end of fermentation. Higher amount ($p>0.05$) of citric acid at end of fermentation (pH 4.5) was observed in JGTY when compared to MGTY and PY.

The addition of MGT into milk may be viewed as stimulatory to citric acid utilization by the yogurt bacteria. The decrease in citric acid, due to its role as substrate during fermentation (Erbaş et al., 2006) by *L. Acidophilus* has been described (Ostlie, Treimo, & Narvhus, 2005) resulting in the accumulation of acetone and diacetyl (Mugula, Nnko, Narvhus, & Sorhaug, 2002).

Despite the minimization of organic acid loss during harvest, storage and analysis, complete prevention of loss not possible. Therefore, changes and reactions in herbs physiology affect organic acid content. Furthermore, As pointed out in earlier studies (Poyrazoglu, Gokmen, & Artik, 2002) cultivar-specific characteristics and environmental factors play very important role in affecting the organic acid content so this may be explain the higher citric acid in JGT + milk than in MGT + milk.

5.3.6.b Lactic acid

Lactic acid is the predominant organic acid in dairy products, produced as a result of

exclusive LAB fermentation of hexoses (Xu, Boylston, & Galtz, 2005). Lactic acid can be used for products that potentially have very large-volume uses in industrial applications and consumer products. It is usually used as a pH adjusting ingredient or as a preservative (either as antioxidant or for control of pathogenic micro-organisms) (John, Nampoothiri, & Pandey, 2008).

However, over acidification a common problem in fermented milk can occur when there is high lactic acid production activity (Guzel-Seydim, Seydim, Greene, & Bodine, 2000). The final concentration of lactate is therefore regarded appropriate at about 8000 mg kg^{-1} with the pH between 4.2 and 4.4 in order for the sensory qualities of sourness and firm coagulum to be satisfactory (Narvhus & Jakobsen, 1996). The concentration of lactate produced in the present studies was enhanced by green tea compared to plain yogurt (Table 5.5). Lactate production in MGT and JGT increased during the 5 h fermentation of milk to 5057.54 and 3741.64 mg L^{-1} for MGTY and JGTY respectively, which were higher ($p<0.05$) than plain yogurt (3100.98 mg L^{-1}).

These values are lower than other studies in yogurt (14550 mg kg^{-1} (Marsili, Ostapenko, Simmons, & Green, 1981) kefir (6400 mg kg^{-1} , (Guzel-Seydim et al., 2000) and probiotic fermented milk (4-14 mg kg^{-1} , (Hilde M. Ostlie et al., 2005) but this may be explained by the differences due to bacterial strain and extent of bacterial inoculation used.

5.3.6.c Acetic acid

Each lactic acid bacteria and propioni bacteria produce mainly lactic acid and propionic acid respectively, but both also produce acetic acid (Xu et al., 2005). Formation of acetic acid in fermented dairy products is probably the result of lactose, citric and lactic acid metabolism of LAB (Mcsweeney & Sousa, 2000) or the catabolism of amino acids

(Tavaria, Dahl, Carballo, & Malcata, 2002). Increment in acetic acid level was seen throughout the fermentation process of milk (Vinderola, Costa, Regenhardt, & Reinheimer, 2002). Too much acetic acid may contribute to a vinegary taste which is considered as one of the disadvantages of fermented milk by bifidobacteria (Nguyen et al., 2012). Thus, from organoleptic perspective, it is important that the level of acetic acid should not be too high in fermented products (Ostlie, Treimo, & Narvhus, 2005). Nonetheless, there is no published standard for acceptable acetic acid level in fermented milks.

Green tea may have beneficial effect with regard to acetic acid level in yogurt (see Table 5.5). The concentration of acetic acid increased slowly with time until a maximum value of 232.05 and 204.11 mg L^{-1} in MGTY and JGTY respectively at the end of fermentation. Plain yogurt also followed similar trend in increased acetic acid with fermentation time, but the amount was higher (269.34 mg L^{-1}) than those found in both green tea yogurts at the end of fermentation. The metabolism of carbohydrates by bifidobacteria was shown to produce acetic and lactic acid in a molar ratio of 3 to 2 (Modler, McKellar, Goff, & Mackie, 1990). Thus the introduction of bifidobacteria in the starter culture with lactic acid bacteria resulted in a higher content of acetic acid than that with lactic acid bacteria during fermentation (Wang, Provan, & Helliwell, 2003). The presence of bifidobacteria in yogurt may therefore produce non-appealing vinegar yogurt taste. This could be made worse by the noticeable amount of acetic acid at the beginning of fermentation (see Table 5.5), as evident by the fact that green tea by itself can be an excellent substrate for acetic acid production (Jayabalan, Marimuthu, & Swaminathan, 2007). However the finding from the present studies appears to suggest that the presence of green tea phytochemicals could selectively inhibit excess acetic production by mixture of LAB and bifidobacteria.

5.3.6.d Pyruvic acid

Pyruvate is the buffered form of pyruvic acid. It is a ketone, and plays an essential role in supplying energy to the cells. Pyruvic acid is an intermediate compound in different metabolic pathways and is toxic in high concentration (Fernandez-Garcia & McGregor, 1994). Pyruvic acid is used directly as an H-acceptor (Guzel-Seydim et al., 2000) making it preferentially converted to lactate. Further, its residual is converted to acetaldehyde and diacetyl (Beshkova, Simova, Frengova, Simov, & Dimitrov, 2003). The amount of pyruvic acid content in milks immediately after inoculation (0 h) was not different between green tea and plain fermented milks (60.13, 57.45 and 55.10 mg L^{-1} , Table 5.5). This acid content increased during fermentation and reached 205.52, 159.27 and 152.29 mg L^{-1} for MGTY, JGTY and PY respectively at the end of fermentation. The marginal differences in pyruvic acid in all three yogurt treatments suggest absence of effects of addition of green tea on glucose metabolism leading to pyruvic acid formation. Increases, as well as decreases, in the concentration of pyruvic acid were observed by Adhikari, Mustapha, & Grun (2003) during milk fermentation cultured with different encapsulated and non-encapsulated bifidobacteria. It would be interesting to find out in more detailed future studies whether green tea, particularly MGT, has any significant influence on glycolytic pathway leading to more pyruvic acid, and subsequently lactic acid, in cultures containing bifidobacteria.

5.3.6.e Propionic acid

LAB obtain the energy they need mostly from lactose producing lactic acid as the main by product whereas acetic, butyric and propionic acids are produced in minor amounts (Yaygin, 1999). Higher amount of propionic acid may be produced in other fermented food

such as Kishk (Tamime & Robinson, 1999) or when the B. Bifidum fermentation condition was in solid state (water content of media at 54.5% and 68.8%) rather than submerged (water content of medium in excess of 90%; Han et al., 2005). MGT contribute about 100 mg L^{-1} whereas JGT contribute about 10 mg L^{-1} into inoculated milk. Both JGTY and PY treatment showed decreasing content of propionic acid as fermentation progressed towards pH4.5 (22.13 and 10.96 mg L^{-1} respectively. On the other hand MGTY treatment showed increasing amount of propionic acid content with fermentation time, producing 341.5 mg L^{-1} at the end of incubation. There are not many reports on propionic acid formation during production of fermented milk.(Guzel-Seydim et al., 2000) did not detect propionic acid in kefir milk and during fermentation of kefir. Propionic acid was also not detected in traditional yogurt and yogurt containing *L.paracasei* (Kristo, Biliaderis, & Tzanetakis, 2003).

5.3.6.f Succinic acid

Succinic acid is one of the secondary metabolites of LAB. Axelsson (1998) proposed a pathway for succinic acid production in heterofermentative lactobacilli growing on glucose and citrate, and the end-products formed are lactate, acetate, carbon dioxide and succinate. As shown in Table 5.5 the amount of succinic acid in milks at the first hour of fermentation was 92.1, 102.23 and 96.46 mg L^{-1} for MGT, JGT and plain fermented milk respectively. This acid increased by 314, 257 and 252% during fermentation for MGTY, JGTY and PY respectively to yield succinic acid concentration of 381.99, 365.33 and 339.43 mg L^{-1} respectively at the end of fermentation. While addition of JGT did not further affect succinic acid production during yogurt formation compared to PY, MGT appeared to cause small increase in succinic acid.

While the concentrations of major organic acids mentioned above increased during milk fermentation, others (i.e chlorogenic acid, caffeoquinic acid, caffeic acid, quinic acid, cinamic acid and 2,5-DHBA) remained unchanged.

Table 5.5 Effects of green tea on organic acid production in yogurt during fermentation of milk

Organic acid	**Sample**	**0 h**	**1 h**	**2 h**	**3 h**	**4 h**	**5h**
2,3-DHBA	MGTY	0.46	ND	ND	ND	ND	ND
	JGTY	0.24	0.13	0.06	1.11	1.22	0.77
	PY	ND	ND	ND	ND	ND	ND
2,5-DHBA	MGTY	2.74	0.08	1.29	0.70	0.76	0.84
	JGTY	2.41	2.79	1.63	ND	ND	ND
	PY	ND	ND	ND	ND	ND	ND
Acetic Acid	MGTY	109.65	131.56	174.49	195.88	224.36	232.05
	JGTY	117.43	127.42	151.33	188.56	199.42	204.11
	PY	79.28	55.19	88.73	142.85	166.86	269.34
Pyruvic Acid	MGTY	60.13	69.00	142.77	181.56	203.58	205.52
	JGTY	57.45	61.38	139.09	121.13	148.41	159.27
	PY	55.10	69.68	119.60	131.44	103.55	152.29
Caffeic Acid	MGTY	1.98	1.20	1.09	1.37	0.20	1.51
	JGTY	0.71	0.56	0.13	0.31	ND	ND
	PY	ND	ND	ND	ND	ND	ND
Caffeoquinic Acid	MGT	0.62	0.30	0.17	ND	1.68	ND

	JGT	ND	ND	ND	ND	ND	ND
	PY	ND	ND	ND	ND	ND	ND
Chlorogenic Acid	MGT	3.38	2.43	2.24	1.46	1.56	1.77
	JGT	0.10	0.10	0.15	0.08	0.09	0.07
	PY	ND	ND	ND	ND	ND	ND
Cinnamic Acid	MGTY	33.89	34.33	21.15	32.43	34.16	33.62
	JGTY	32.45	34.44	29.34	27.81	34.78	34.33
	PY	30.16	30.23	32.03	35.74	33.40	33.08
Citric Acid	MGTY	337.63	276.59	229.03	182.79	165.80	132.97
	JGTY	367.66	327.47	344.21	315.23	259.05	172.23
	PY	300.82	291.02	277.9	249.59	224.19	131.11
Coumaric Acid	MGTY	1.93	1.23	1.24	0.83	1.04	1.08
	JGTY	0.74	0.82	0.50	0.51	0.68	0.61
	PY	ND	ND	ND	ND	ND	ND
Lactic Acid	MGTY	2095.07	2206.77	2860.83	3657.05	4527.76	5057.54
	JGTY	2341.24	2337.15	2774.89	3268.58	3517.41	3741.64
	PY	2147.72	2300.03	2399.13	2567.41	2795.00	3100.98
Propionic Acid	MGTY	139.25	137.68	190.02	234.24	259.69	341.50
	JGTY	43.35	34.32	20.29	20.42	21.01	22.13
	PY	31.87	26.73	13.45	12.03	11.38	10.96
Quinic Acid	MGTY	18.15	11.95	19.09	20.32	16.09	14.57
	JGTY	4.31	6.48	11.65	10.12	9.39	11.06
	PY	ND	ND	ND	ND	ND	ND

Succinic Acid	MGTY	92.10	139.86	162.55	193.32	232.19	381.99
	JGTY	102.23	116.91	158.78	222.12	243.51	365.33
	PY	96.46	156.67	222.63	320.92	325.60	339.43

5.3.7 Effects of green tea on proteolysis of milk protein during fermentation of milk

During fermentation, milk proteins are hydrolysed by extracellular proteinases produced by lactic acid bacteria resulting in an increase in the amount of free amino groups as quantified by the OPA method (Donkor, Henriksson, Vasiljevic, & Shah, 2006). The OPA-based spectrophotometric assay reacts with the α-amino groups, thus giving an indirect measurement of proteolytic activity. Figure 5.4 shows the concentration of water soluble protein with free α-amino groups (OPA value) in the fermented milks during the 360 min of incubation at 41˚C. The OPA values for inoculated milks in the absence or presence of green tea water extract were similar during the first 120 min of incubation. However increased in proteolysis in green tea yogurts occurred after the 120 mins of incubation (7.77, 7.12 and 6.27 mg/ml for, JGTY, MGTY and PY respectively). The extent of proteolysis in yogurt was highest ($p< 0.05$) in MGTY followed by JGTY and PY at the end of fermentation (22.01, 18.58 and 7.45 mg/ml respectively). Lactic acid bacteria are fastidious organisms that require an external source of amino acids or peptides that are provided by the most abundant and proline-rich milk proteins, caseins (Savijoki et al., 2006). The increased proteolytic activities in green tea yogurts illustrate an enhanced microbial growth and metabolism, and thus an increase in OPA values with time was consistent and significant throughout the fermentation. Higher proteolysis in green tea yogurts suggest favorable conditions induced by the green tea which promote the growth and release of proteinases by lactic acid bacteria. The availability of highly digestible

protein, thus source of nitrogen coupled with readily available energy from degradation of milk sugar lactose (formation of organic acids, see Table 5.5) encourage the proliferation of LAB (see Section 5.3.5 on TA-VCC relationship).

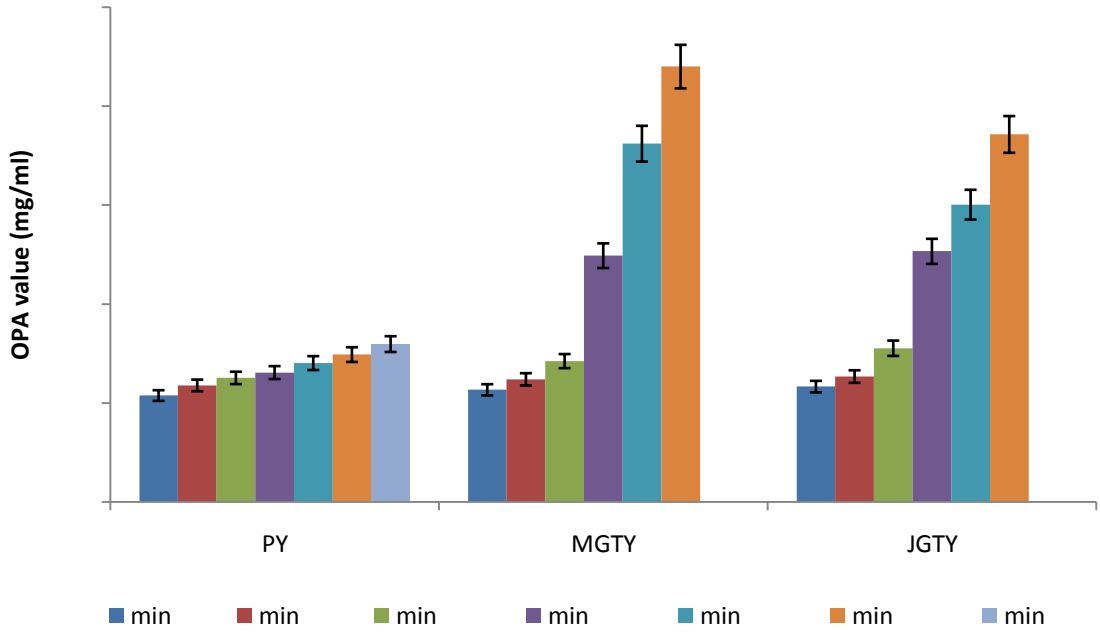

Figure 5.4. Changes in o-phthalaldehyde (OPA) values in plain- and green tea-yogurts during fermentation (41 ˚C). PY=plain yogurt, MGTY=Malaysian green tea yogurt and JGTY= Japanese green tea yogurt. Each experiment was repeated three times and values are means ± SD.

5.4 Conclusion

The present study demonstrated a profound effect of green tea on the increase in antioxidant capacity, microbial growth and milk protein proteolysis. Green teas had direct effect stimulating the growth of *Lactobacillus spp.* and *S. thermophilus* during fermentation of milk. Increased microbial growth as a result of addition of green tea resulted in increased

microbial metabolic activities as reflected in increased TA and proteolysis (OPA values). Both green tea yogurts contained higher antioxidant capacities than plain yogurt but the increase was not explicitly due to increased in microbial growth during fermentation of milk. Green tea can thus be used to enhance microbial growth and increase the antioxidant capacity of yogurt.

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