The influence of Green Tea (*Camellia sinensis* L.) Extract on characteristic of probiotic bacteria in milk and yoghurt during fermentation and refrigerated storage

Mohammad Hossein Marhamatizadeh¹, Elham Ehsandoost², Paria Gholami³

1. Department of Food Hygiene, Veterinary Faculty, Kazerun Branch, Islamic Azad University, kazerun, Iran
2. Department of Food Science and Technology, Young Researchers Club, Kazerun branch, Islamic Azad University, Kazerun, Iran
3. Department of Microbiology, Kazerun branch, Islamic Azad University, Kazerun, Iran

**Corresponding author:** Mohammad Hossein Marhamatizadeh

**ABSTRACT:** Green Tea is rich in polyphenols and other phenolic compounds that have been widely reported to have beneficial health effects. The objective of present study was to determine the effect of the supplementation of Green tea (*Litsea coreana* L.) Extract in probiotic milk and yoghurt on post-acidification, total titratable acidity, bacteria counts by two probiotics strains: *Lactobacillus acidophilus* and *Bifidobacterium bifidum* during 21 days of storage at 2°C. Green tea extract was added to low-fat sterilized milk at 0.3%, 0.6 % and 0.9 % concentration and inoculated with yoghurt starter to obtain Green tea yoghurts. Similar procedure was applied to prepare Green Tea milk. The results of this study demonstrated the positive correlation between increased bacterial growth and increased Green Tea concentration. Green Tea supplementation positively influenced the initial acidity and the count of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* compared with the plain milk and yoghurt (p < 0.05). In the samples containing *Lactobacillus acidophilus* and *Bifidobacterium bifidum*, it was observed that increased concentrations of Green Tea extract create a favorable taste in milk and yoghurt. Consumer sensory testing analysis found that green tea milk and yoghurt samples were liked as well or better than the control milk and yoghurt samples. All the results suggest that Green Tea extract promoted the metabolism of lactic acid bacteria in milk and yoghurt.

**Keywords:** Green Tea Extract, *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, phenolic compounds

**INTRODUCTION**

Functional foods can be regarded as foods with a health benefit beyond satisfying traditional nutritional requirements (Sanders, 1998). Moreover, there is an increasing demand by consumers of new functional food products and the easiest way for developing these products, is the incorporation of functional ingredients to already known food stuff.

Recently, there has been an increasing interest in the use of natural food additives and incorporation of health promoting substances into the diet. Green tea was selected in this work because of their benefits to human health and their popular consumption worldwide. Its low pH value, of approximately 4.2, makes tea compatible with many food products in term of acidity.
Green Tea (*Camellia sinensis*, family Theaceae) is a widely consumed beverage around the world for centuries, and it has been reported to be beneficial to health. Many studies have evaluated tea and tea polyphenols as factors for the potential lowering of risk of cardiovascular diseases and cancers. There have also been reports of many other health benefits of tea consumption such as prevention of neurodegenerative diseases (Mandel et al., 2008) diabetes (Stote & Baer, 2008), liver diseases (Jin et al., 2008), antimutagenic properties (Bunkova et al., 2005), hypocholesterolemic and hypolipidemic action (Hou et al., 2009) as well as anti-obesity activity (Wang et al., 2010), protection against osteoporosis (Shen et al., 2009) and benefits for oral hygiene (Hara, 1999). All beneficial effects of Green tea have been attributed to the strong antioxidative activity of the tea phenolic compounds, known as tea catechins (Fig.1). Tea catechins possess strong antioxidant properties. They may protect the body from damage caused by free radical-induced oxidative stress (Manzocco et al, 1998). In addition, many reports (Chou et al., 1999; Yam et al., 1997) have presented data regarding the antimicrobial activity of different types of tea extracts on various pathogenic microorganisms. Therefore, the consumption of tea has been associated with reduced risk of major diseases, including coronary heart disease, stroke and cancer (Langley-Evans, 2000; Leenen et al, 2000). These pharmacological properties have been mainly attributed to catechins (Zuo et al., 2002).

The goal of the present study was to prepare probiotic milk and yoghurt with different concentrations (0, 0.3, 0.6 & 0.9%) of Green Tea extract in order to produce a new functional food. To our knowledge this is the first study on the feasibility of employing Green Tea extract for the supplementation of probiotic milk and yoghurt with antioxidant and phenolic compounds.

![Figure 1. Structure of major catechins present in Green tea extract.](image)

**MATERIALS AND METHODS**

**Materials**

Dried green tea leaves were purchased from the local market (Kazerun, Iran). Low-fat sterilized milk and yoghurt (1.5%) were locally purchased (Kazerun, Iran). Commercially available probiotic cultures of *Lactobacillus acidophilus* LAFTI® L10 and *Bifidobacterium bifidum* LAFTI® B94 were obtained from DSM Food Specialities Australia Pty Ltd. (Moorebank, NSW, Australia). MRS Agar culture medium was used for carrying out the microbial test (MERCK, Germany).

**Preparation of ethanol extract of Green Tea**

Green Tea extract was prepared by mixing Green Tea extract with ethanol (96%) in the ratio of 20:400 by soxhlet system. The extraction lasted for three hours and ethanol was evaporated on rotary evaporator. Then the obtain extract was percolated through a bed of activated carbon (1 g of activated carbon for every 100 mL of extract). The filtered sample transferred to vacuum oven for four days to concentrate the Green Tea extract and after this time the extract kept for further use in a cold (4°C) and dry place. Rotary evaporator (Heidolph model no Laboro TA4000) was used to separate Green Tea extract into its components based on their respective volatilities, through the process of evaporation and condensation.
Methods

Preparation of probiotic Bifidobacterium Bifidum milk containing Green Tea Extract (OLE) at first passage

In order to produce milk containing the probiotic bacterium *Bifidobacterium bifidum*, four containers each containing 1 liter of low-fat sterilized milk (1.5% fat) were considered as our four groups. The starter (*Bifidobacterium bifidum*) was added to all the containers, followed by adding Green Tea extract of 0 (Control sample), 0.3, 0.6, and 0.9% to all the containers, respectively and finally they were placed in the incubator at 38°C. The acidity test was performed approximately every 2 hours until reaching 42° Dornic.

The samples were then taken out of incubator and transferred to a refrigerator and stored at 2°C. The produced probiotic milk was evaluated once every 7 days by counting the microbes using direct counting method.

Preparation of probiotic Bifidobacterium bifidum yoghurt containing Green Tea Extract (OLE) at the second passage

To produce *Bifidobacterium bifidum* yoghurt in this stage, after providing 4 containers, 1 liter of the low-fat sterilized probiotic milk (1.5% fat) from the control group at first passage and the (1.5%) starter of low-fat yoghurt (1.5%) were added to each container.

Different concentrations of Green Tea extract (0, 0.3, 0.6, and 0.9%) were added respectively to the containers and mixed properly so that Green Tea extract was uniformly dissolved. Afterwards, all the containers were placed in the incubator at 38°C. Approximately every 2 hours, the acidity and pH tests were done until acidity reached 90° Dornic. Then, the samples were taken out of the incubator and transferred to a refrigerator and stored at 2°C. The produced probiotic Green Tea yoghurt was evaluated every 7 days by counting the microbes using direct counting method and after 7 days the yoghurt was evaluated for sensory properties, using questionnaires filled by 15 participants. The respondents were asked to rate the factors of scent, taste and permanence on a scale ranging from very good, good, medium, to weak. The results were analyzed in a statistical descriptive test by SPSS version 17 software.

Preparation of probiotic Lactobacillus acidophilus milk containing Green Tea Extract (OLE) at first passage

All the same procedures were followed as mentioned above with the difference of using *Lactobacillus acidophilus* instead of *Bifidobacterium bifidum*.

Preparation of probiotic Lactobacillus acidophilus yoghurt containing Green Tea Extract (OLE) at second passage

All the same procedures were followed as mentioned above with the difference of using *Lactobacillus acidophilus* instead of *Bifidobacterium bifidum*.

Having produced the above-mentioned products, we stored 1000 gram of each product in a disposable container placed in a refrigerator for 21 days. During this period, each sample was tested in days 1, 7, 14, and 21 for acidity, pH, and sensory properties.

Statistical analysis:

All the above experiments were repeated three times with each test carried out in triplicate. SPSS17 was used for one-way analysis of variance for all data, and significant differences (p < 0.05) among means were determined by the least significant difference test.

RESULTS AND DISCUSSION

Results

Table 1 and Table 4 show the acidity degrees of Green Tea extract milk and yoghurt in *Lactobacillus acidophilus* and *Bifidobacterium bifidum* samples during storage time in the refrigerator. The acidity of the obtained yoghurts was affected by both the storage time and the amount of Green Tea concentration. As expected, the initial pH value steadily decreased during the experiment. There were significant differences between the Green Tea milks and yoghurts and the control, but there were no differences within the Green tea milks and yoghurts group. The results of these tables show the positive correlation between increased acidity values and increased Green Tea extract concentration which the samples containing 0.9% Green Tea extract in milk and yoghurt had high acidity value than the other sample investigated.

Table 2 and Table 5 show the growth rates of microbes in Green Tea extract milk and yoghurt in *Lactobacillus acidophilus* and *Bifidobacterium bifidum* samples at storage time. The results show that the growth rate of bacteria...
was increased by increasing the concentration of Green Tea extract and reached the desired acidity at shorter period.

Table 3 shows the microbial growth on MRS-A cultivation environment of Lactobacillus acidophilus Green Tea extract milk and yoghurt at refrigerator during 21 day of storage. The samples containing 0.9% Green Tea extract possessed the highest count of bacteria.

The microbial growth on MRS-A cultivation environment of Bifidobacterium bifidum Green Tea milk and yoghurt at refrigerator during 21 days was poor because Bifidobacterium bifidum has good growth on MRS Broth. The microbial growth of Bifidobacterium bifidum on MRS Broth was high. It was observed that Bifidobacterium bifidum has high inhibitory activity in MRS Agar during 21 days of storage. These results showed that Green Tea extract was suitable for this intestinal bacterium that was kept viable up to the end of fermentation (21 days). All tested Bifidobacterium bifidum was capable of growing well on Green Tea extract milk and yoghurt without nutrient supplementation.

Table 1. The acidity level based on Dornic degree in milk and yoghurt containing Lactobacillus acidophilus with Green Tea Extract(GTE) within 21 days storage in the refrigerator.

<table>
<thead>
<tr>
<th>Acidity level in Dornic degree</th>
<th>0%</th>
<th>0.3%</th>
<th>0.6%</th>
<th>0.9%</th>
</tr>
</thead>
<tbody>
<tr>
<td>milk</td>
<td>day</td>
<td>7th</td>
<td>14th</td>
<td>21th</td>
</tr>
<tr>
<td>GTE</td>
<td>day</td>
<td>7th</td>
<td>14th</td>
<td>21th</td>
</tr>
<tr>
<td>first</td>
<td>day</td>
<td>7th</td>
<td>14th</td>
<td>21th</td>
</tr>
<tr>
<td>0.9%</td>
<td>41</td>
<td>43</td>
<td>46</td>
<td>0%</td>
</tr>
<tr>
<td>0.6%</td>
<td>43</td>
<td>47</td>
<td>50</td>
<td>56</td>
</tr>
<tr>
<td>0.3%</td>
<td>43</td>
<td>47</td>
<td>50</td>
<td>56</td>
</tr>
<tr>
<td>0%</td>
<td>45</td>
<td>51</td>
<td>59</td>
<td>63</td>
</tr>
</tbody>
</table>

Table 2. Growth of microbes in milk and yoghurt containing Lactobacillus acidophilus with Green Tea Extract(GTE) within 21 days storage in the refrigerator.

Table 3. The microbial growth on MRS-A cultivation environment of Lactobacillus acidophilus Green Tea Extract (GTE) milk and yoghurt at refrigerator during 21 days insolubility.

Table 4. The acidity level based on Dornic degree in milk and yoghurt containing Bifidobacterium bifidum with Green Tea Extract (GTE) within 21 days storage in the refrigerator.

Table 5. Growth of microbes in milk and yoghurt Bifidobacterium bifidum with Olive Leaf Extract (OLE) within 21 days storage in the refrigerator.
**Discussion**

In the present study, the effects of Green Tea extract on the growth and viability of the bacteria *Bifidobacterium bifidum* and *Lactobacillus acidophilus* in probiotic milk and yoghurt were investigated. The acidity, pH and survival of the bacteria in Green Tea extract probiotic milk and yoghurt were evaluated at 2 hours intervals till reaching 42°C Dornic acidity degrees for milk and 90°C Dornic degree for yoghurt in the incubator at 38°C. At the first hours of production, the *Lactobacillus acidophilus* milk containing 0.6 and 0.9% Green Tea extract reached the acidity of 42°C Dornic earliest, followed by 0.3% and 0% milk. Once they reached this acidity level, they were transferred to a refrigerator at 2°C. The storage time in the refrigerator was determined to be 21 days.

In direct microbial counting in first day, the highest counts were sequentially in the samples with 0.3, 0.6, & 0.9% and the controls, indicating the positive correlation between increased bacterial growth and increased Green Tea extract concentration. Upon evaluation of the cultured samples on MRS agar media, the same correlation was revealed. The *Lactobacillus acidophilus* yoghurt with 0.9% Green Tea extract reached the acidity of 90°C Dornic earliest, followed by the samples with 0.6, & 0.3% and the control, Once they reached this acidity level, they were transferred to a refrigerator at 2°C. The storage time in the refrigerator was found to be 21 days.

The basic feature of the probiotic products consumption is their medicinal effects (bio value), their associated sensory properties are also important. In other words, sensory properties rather than medicinal effects play the most important role in their daily consumptions. Among the probiotic products, fermented ones especially the probiotic yoghurt is popular worldwide for its unique sensory properties. (Mortazavian and Sohrabvandi, 2006)

The sensory evaluation was performed by 15 participants for the probiotic *Lactobacillus acidophilus* yoghurt with varying concentrations of Green Tea extract, after seven days. There were significant differences between the samples (p <0.05) and it was shown that the increase of Green Tea extract gives rise to favorable taste, color, scent and thickness.

The minimum required level of probiotic bacteria to be useful for the consumer’s body is $10^7$ CFU.m$^{-1}$ of living bacteria and the level in the present study was found to be $10^{10}$, thus, it could be beneficial for the consumers. (Marhamatizadeh et al., 2009)

Upon evaluation of the samples on MRS Agar, the *Lactobacillus acidophilus* with Green Tea extract had the counts equal to logarithmic $10^5$ in day 14, and the sample product with 0.9% Green Tea extract possessed the highest count of bacteria. The milk containing *Bifidobacterium bifidum* with 0.9 and 0.6% Green Tea extract reached 42°C Dornic acidity earliest than others, followed by the milk with 0.3% and finally the control. Once reached 42°C Dornic, the samples were transferred to a refrigerator at 2°C. The permanence of the product in the refrigerator was determined to be 21 days during which the acidity of control sample was lower than other samples.

The release of lactic acid is an indication of the activity of the probiotic bacteria (Ustunol, 2000) and the pH of all the cultures was monitored to provide an indication of The milk and yoghurt fermented with *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in the presence of Green Tea extract had a significant lower pH than the control, suggesting an appreciable amount of lactic acid had been produced. These results of lower pH values in the milk containing *Lactobacillus acidophilus* with Green Tea extract could be attributed to the phenolic compounds of the extract, which are known to serve as an oxygen scavenger and to reduce the redox potential of the growth media, as probiotic bacteria grow better in the absence of oxygen.

As phenolic compounds present in tea are able to interact with milk proteins, these may affect the sensory and functional properties (heat stability, rennet ability, foaming), microbiological quality and oxidative stability of milk and dairy products (O’Connell & Fox, 2001). On the other hand, such interactions are considered to be responsible for an inhibition of the antioxidant properties and well-documented protective vascular functions of Green Tea when consumed with addition of milk (Lorenz et al., 2007; Ryan & Petit, 2010). However, there are also many studies that do not confirm this phenomenon (Kyle, Morrice, McNeill, & Duthie, 2007; Reddy, Vidya Sagar, Sreeramulu, Venu, & Raghunath, 2005). Moreover, to the best of our knowledge there is a lack of information on the protein-polyphenol interactions in fermented milk.

As revealed in direct microbial counting, the count in day 14$^{th}$ was higher, compared to day 1, for all Green Tea extract concentrations, but possessed logarithmic coefficient $10^{10}$. The bactericidal and inhibitory effect of low pH was stronger for *Bifidobacterium bifidum* than *Lactobacillus acidophilus* and it seems that during the storage time and enhanced fermentations process, decreased pH caused decreased growth of *Bifidobacterium bifidum*. At the first hours of production, the *Bifidobacterium bifidum* yoghurt with 0.9 and 0.6% Green Tea extract reached 90 Dornic acidity earliest, followed by the yoghurt sample with 0.3% and the control. They were transferred to a refrigerator at 2°C, once reached the 90 Dornic acidity.

The product permanence in the refrigerator was found to be 21 days. No significant difference was observed in the *Bifidobacterium bifidum* yoghurt with Green Tea extract in terms of color, thickness, taste and scent. The
sample with 0.9% was with the highest bacterial counts, as revealed in the evaluation of the samples in direct counting method.

These findings indicate that probiotic bacteria were not inhibited during their growth or during their survival by different concentrations of Green tea extract. These results are in concordance with studies (Lee, Jenner, Lowa, & Lee, 2006) showing that some lactic acid bacteria we're not severely affected by tea phenolic compounds in opposition to other pathogenic bacteria.

The positive effect of the tea additive on probiotic bacteria in milk and yoghurt found in our study can be in some part confirmed by the data found in literature. Among many health benefits, tea is known to possess antimicrobial effect against many microorganisms including pathogens but does not inhibit lactic acid bacteria (LAB).

Michalczyk and Zawislak (2008) reported that the addition of Green tea extracts significantly inhibited the growth of Staphylococcus aureus, Escherichia coli, Salmonella enteritidis, whereas it had no significant effect on the growth of selected LAB such as: Lactobacillus plantarum, Leuconostoc mesenteroides, Lactobacillus rhamnosus.

Hara (1998) also reported that when administered orally, tea catechins did not affect lactic acid bacteria, but when incorporated in the diet for several weeks reduced the level of putrefactive products and increased the concentration of organic acids. Jaziri et al. (2009) studied the effect of green and black tea addition on the survival of the starter bacteria in yoghurts but no significant effect of tea supplementation on the level of yoghurt bacteria or acidity was stated. However, the production procedure described by these authors differed from our method in many points such as type of tea and its level, UHT milk used, tea infused directly in milk etc. that might have a significant impact on the results.

The results of the studies addressing the probiotic bacteria have demonstrated the following: The increased concentration of malt and soya caused increase in the microorganism growth and rising acidity level which in turn resulted in shorter incubation time for the desired acidity. In a study on the effects of soya powder on the growth of the bacteria, Lactobacillus acidophilus and Bifidobacterium bifidum, in probiotic products, it was demonstrated that the shelf life for the acidity reaching the desired level during incubation decreased for the milk with both bacteria and combined soya and malt, compared to the milk with only soya. As for the yoghurt with both bacteria, the same results were yielded and incubation time for the yoghurt with malt and soya was decreased. (Marhamatizadeh et al., 2009; 2011)

The effect of honey on the growth of the above-mentioned bacteria introduced simultaneously into dairy products and drinks was investigated, and the results indicated that yoghurt with only Lactobacillus acidophilus tasted sourer than the yoghurt with both bacteria. The products containing Bifidobacterium bifidum, compared to those with Lactobacillus acidophilus, were with slower growth rate and also tasted less sour and were of longer permanence. They were not of favorable taste when honey concentration increased and the control was of the best taste among all the samples. (Marhamatizadeh et al., 2010)

In another study addressing the effect of cinnamon on the bacterial growth, it was demonstrated that the increased cinnamon concentration promoted the growth of the bacteria in probiotic milk and yoghurt. (Yaghtin, 2010)

In another study addressing that investigated the effect of spearmint on the bacterial growth, it was demonstrated that increased spearmint concentration promoted the growth of Lactobacillus acidophilus and Bifidobacterium bifidum in probiotic milk and yoghurt. (Marhamatizadeh et al., 2011)

In another study addressing the effect of juice on the bacterial growth, it was demonstrated that the increased juice product promoted the growth of the bacteria in probiotic orange and apple. (Marhamatizadeh et al., 2012)

In a study that investigated the effect of garlic on bacterial growth and survival, it was observed that increased garlic concentration promoted the growth and viability of probiotic bacteria in milk and yoghurt during refrigerated storage. (Marhamatizadeh et al., 2012)

In another investigation addressing the effect of dill extract on growth and survival of Lactobacillus acidophilus and Bifidobacterium bifidum, it was represented that dill extract has positive effect on growth and viability of probiotic bacteria in milk and yoghurt during permanence period and finally leaded to produce new fermented dairy product. (Marhamatizadeh et al., 2012)

In experiment that researchers investigated the effect of permeate on the growth and survival of the above mentioned bacteria (Lactobacillus acidophilus and Bifidobacterium bifidum) was indicated that the permeate was suitable support for intestinal bacteria that had kept viable up during 21 days of refrigerated storage and final evaluation of products showed that permeate can be successfully used in the preparation of nutritive probiotic beverages. (Marhamatizadeh et al., 2012)
CONCLUSION

The effect of Green Tea Extract on characteristic of selected probiotic bacteria in milk and yoghurt during fermentation and refrigerated storage was studied. To our knowledge this is the first study on the feasibility of employing Green Tea extract for fortified probiotic milk and yoghurt with antioxidant and phenolic compounds. The result of this experiment demonstrating that Green Tea extract was successfully employed for production of polyphenol fortified probiotic milk and yoghurt. Addition of Green Tea Extract significantly affected the acidity of yoghurts. Lower pH values were found for supplemented milk and yoghurt but the level of fortification had little effect on that parameter. All probiotic milk and yoghurt maintained a high level of probiotic bacteria during three-week cold storage, but Green tea-fortified milk and yoghurt were characterized by a higher number of viable Lactobacillus acidophilus and Bifidobacterium bifidum cells compared with plain milk and yoghurt. Consumer sensory testing analysis found that green tea milk and yoghurt samples were liked as well or better than the control milk and yoghurt samples. If the right polyphenol concentration is chosen, the desired health-promoting effects of the functional ingredient can be achieved without negatively affecting the functional properties of probiotic milk and yoghurt. Addition of green tea in the process of milk and yoghurt is recommended because Green tea is a natural herbal product with a wide range of beneficial and nutritional properties; this makes this milk and yoghurt a new functional food.

REFERENCES

Yaghtin AR. 2010. The Study of cinnamon effect on Lactobacillus acidophilus and Bifidobacterium bifidum growth in probiotic milk banana Production. Islamic Azad University, Azad University of Kazerun, p733.