Food Chemistry 112 (2009) 614–620

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Effect of green and black teas (*Camellia sinensis* L.) on the characteristic microflora of yogurt during fermentation and refrigerated storage

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ARTICLE INFO

Article history:
Received 30 January 2008
Received in revised form 29 March 2008
Accepted 11 June 2008

Keywords:
Green tea
Black tea
Yogurt bacteria
Catechins
Functional food

ABSTRACT

The effect of tea on the fermentation and survival of yogurt microorganisms was studied. Green and black teas were added to milk at the beginning of fermentation. Acidity of yogurt products and survival of their microflora were studied during 42 days at 4 °C. Results showed that the presence of tea did not significantly ($P < 0.05$) influence the yogurt characteristic microorganisms. HPLC studies demonstrated that yogurt bacteria did not affect tea catechins when they were incubated together for 48 h. Indeed, all five products reached about 10^9 CFU/ml after 6 h of fermentation. Viability during 6 weeks storage at 4 °C varied very little (8.35 < log CFU/ml < 8.65). Similarly, green and black teas had no effect on lactic acid levels of the final products (after 6 weeks of storage, acidity remained above 80 °D). According to these findings, addition of teas or tea catechins to yogurt can be recommended to take advantage of their beneficial properties on human health attributed to their antioxidant and antimicrobial activities.

1. Introduction

Tea (*Camellia sinensis*, family Theaceae) is consumed worldwide and is second only to water in its popularity as a beverage (Thangapazham et al., 2007). Many health benefits have been ascribed to consumption of this beverage, including the effects of reduction of cholesterol, protection against cardio-vascular disease and cancer (Zuo, Chen, & Deng, 2002). All beneficial effects of tea have been attributed to the strong antioxidative activity of the tea phenolic compounds, known as tea catechins.

Tea catechins possess strong antioxidant properties, i.e. they may protect the body from damage caused by free radical-induced oxidative stress (Manzocco, Anse, & Nicoli, 1998). In addition, many reports (Chou, Lin, & Chung, 1999; Yam, Shah, & Hamilton-Miller, 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 (Benzie & Szeto, 1999; Langley-Evans, 2000; Leenen, Roodenburg, Tijburg, & Wiseman, 2000; Ramarathnam, Osawa, Ochi, & Kawakishi, 1995; Robinson, Maxwell, & Thorpe, 1997). These pharmacological properties have been mainly attributed to catechins (Zuo et al., 2002).

Yogurt is a well known fermented dairy food, which is usually manufactured from cow's milk with or without the addition of some natural derivatives of milk, and possesses a gel structure that is the result of coagulation of the milk proteins by lactic acid produced by *Streptococcus thermophilus* (*S. thermophilus*) and *Lactobacillus bulgaricus* (*L. bulgaricus*) (Robinson, 2003). Yogurt must contain an abundant and viable microflora of starter origin at the time of consumption, and this is stated in the food laws of many countries, with minimum values ranging between 10^6 and 10^8 CFU/g (Anonymous, 2001).

Recently, there has been an increasing interest in the use of natural food additives and incorporation of health-promoting substances into the diet (Varga, 2006). Green and black teas were 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. Tea catechins have been used as antioxidant compounds in many food matrices such as meats, poultries, fishes and vegetable oils (Yilmaz, 2006).

Since tea has an antimicrobial activity against a large spectrum of pathogenic bacteria (Chou et al., 1999; Hamilton-Miller, 1995; Jaziri & Hamdi, 2005; Yam et al., 1997), its addition to milk before fermentation would protect the final product against pathogenic or undesirable bacteria.

In the present study, we investigate first, the ability of *S. thermophilus* and *L. bulgaricus* to grow and survive in presence of green

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doi:10.1016/j.foodchem.2008.06.017

and black teas (*C. sinensis* L.), then the effect of these bacteria on main tea catechins in order to produce a new functional food.

2. Materials and methods

2.1. Chemicals

All chemicals, organic solvents, and reagents were analytical grade materials. Ethyl acetate (extra pure) was delivered by LabScan (Dublin, Ireland). Acetic acid and methanol (HPLC grade) was obtained from Fischer Chemical (Illkirch, France). Anhydrous sodium sulphate was from Scharlau (Barcelona, Spain). Water used in all the experiments was doubly distilled and deionized using an Ultrapure Organex cartridge (Millipore, Guyancourt, France). Catechin gallate (CG), epigallocatechin (EGC), catechin (C), epigallocatechin gallate (EGCG), gallocatechin gallate (GCG) and epicatechin gallate (ECG) were purchased from Sigma–Aldrich (Steinheim, Germany).

2.2. Sampling

Green and black tea leaves were purchased from a retail market. The tea infusion concentrations used in the various experiments were 2.0% and/or 4.0% (w/v) corresponding respectively to once and twice the strength of a normal "cup of tea" (Yam et al., 1997). Tea infusions for yogurt manufacture were prepared in milk; however, those for HPLC analysis were prepared in water.

2.3. Manufacture and storage of yogurts

UHT-Half fat milk, purchased from a French market, was divided into five equal portions then was taken into ebullition and fortified with green or black teas at levels of 2.0% and 4.0% (w/v). The fifth portion was considered as the blank control. The teas were infused for 10 min then the different batches were filtered through sterile cotton to remove all particles. The process milks thus obtained were cooled to 45 °C and inoculated with 10% (v/v) yogurt culture and divided into 5 × 21 sterile and tightly capped tubes (30 ml). Incubation lasted 6 h at 42 °C to reach a pH value of 4.5–4.6. Thereafter, the yogurts were cooled and stored at 4 °C. The entire experimental program was repeated twice. The process diagram is presented in Fig. 1 (Jaziri, Gannoun, Ben Othman, Ben Slama, & Hamdi, 2005) and shows all the steps of manufacture that could be used in dairy technology.

2.4. Microbiological analysis

Three tubes of all four products were taken at each sampling time, i.e., after 0, 7, 14, 21, 28, 35, and 42 days of refrigerated storage. Samples were aseptically removed from tubes and diluted 10 times into 90 ml of 0.1% peptone water. Further dilutions were made as required. The standard pour plate method was employed to determine the viable cell counts of starter organisms. M17 agar (Difco) was used to enumerate *S. thermophilus*. The pH of the medium was set at 7.1 ± 0.1.

The inoculated plates were incubated at 37 °C for 48 h under aerobic conditions. *S. thermophilus* formed lenticular colonies (Marth Elmer & Steele James, 2001). Colony forming units (CFU), expressed as log per milliliter, were used to report survival of streptococci. The streptococci identified on the basis of colonial type were confirmed by microscopic examination using a binocular microscope (Olympus BX40, Rungis, France). *S. thermophilus* were Gram-positive, spherical or ovoid cells (0.7–0.9 μm in diameter)

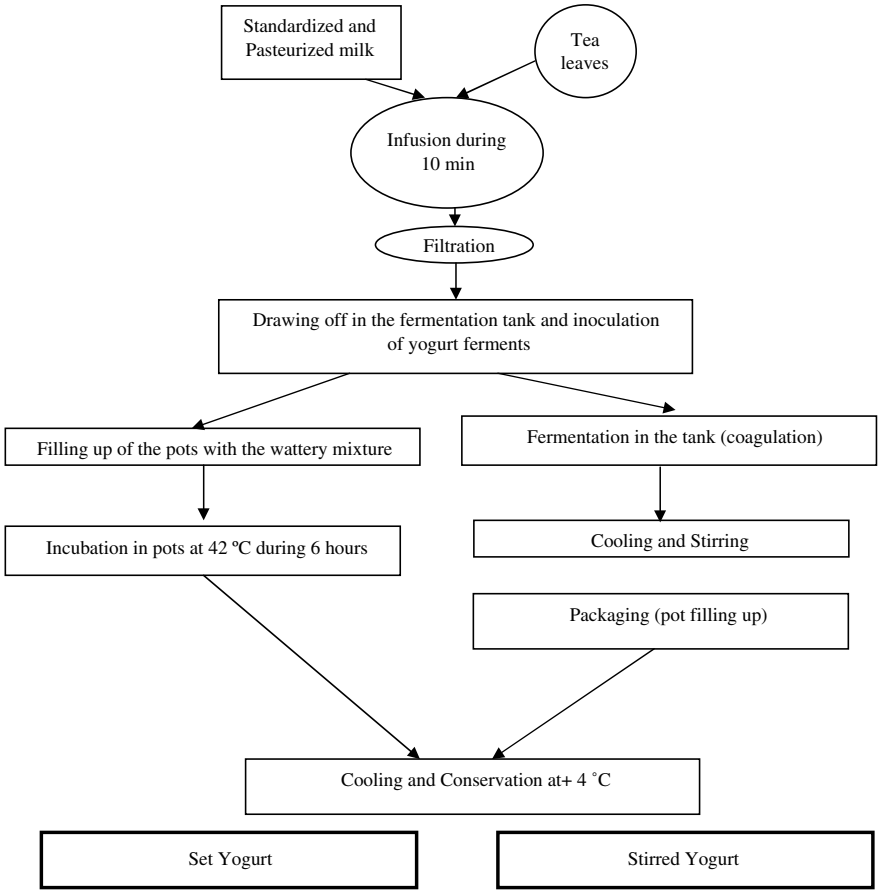

Fig. 1. Diagram of the manufacture of yogurt with tea (Jaziri et al., 2005).

appearing in pairs or in long chains. Acidified MRS agar (Difco) with pH of 6.5 was used for the enumeration of *L. bulgaricus*. The plates were incubated at 37 °C for 72 h. Anaerobic culture jars (3 l) were employed to generate anaerobic conditions, atmospheric oxygen being absorbed by means of (AnaeroGen AN 25 sachets). *L. bulgaricus* formed 1–3 mm diameter colonies (Marth Elmer & Steele James, 2001). The counts were expressed as log CFU/ml. The lactobacilli identified were corroborated by observation under an (Olympus BX40, Rungis, France) microscope. *L. bulgaricus* were Gram-positive, nonmotile, nonsporeforming, generally short rods, which, however, sometimes appeared in longer forms (Marth Elmer & Steele James, 2001).

The initial viable cell counts of starter organisms in the five product formulations were about 3×10^6 CFU/ml. After fermentation of the different products and during all the refrigerated storage period the viabilities of both *S. thermophilus* and *L. bulgaricus* in presence of different concentrations of tea were presented in log CFU at *n* weeks of storage = function (time (days)).

Detection of yeast, fungi, salmonella and sulphite reducing clostridia was carried out during the storage period. Potato Dextrose Agar was used to detect yeast and/or fungi; incubations were carried out at 37 °C for 24 h. Sulphite reducing clostridia detection was carried out using the TSN (Tryptone–Sulphite–Neomycine) broth (Fluka Biochemica, India) in Hungate stoppered tubes, heat-shocked (80 °C, 10 min), and then incubated 24–48 h at 46 °C. For Salmonella detection, Salmonella-Shigella Agar (Oxoid, Hampshire, UK) was used at 37 °C for 48 h. Each experiment was performed in duplicate.

2.5. Acidity measurement

The pH value of samples was determined at room temperature with an inolab 740 pH-meter and combined glass electrode (Hanna Instruments Deutschland, Germany) standardized with pH 4.01 and 7.01 standard buffer solutions (Scharlau chemie S.A, Barcelona, Spain).

Samples (10 ml) were titrated using NaOH (0.1 N). The amount of NaOH used (expressed in milliliter) was multiplied by 10, and titratable acidity was thus obtained in Dornic degrees (°D) or in gram of lactic acid per litre (Dilmi-Bouras, 2006).

2.6. Preparation of bacteria inocula

L. bulgaricus and *S. thermophillus* were isolated from a commercialised fresh yogurt culture. Cells of *L. bulgaricus* and *S. thermophillus* were respectively cultivated on MRS and M17 broth thereafter, they were harvested by centrifugation at 6000 rpm for 10 min after incubation at 37 °C during 18 h. The cells pellets were then washed twice with sterile saline water (9‰, w/v) and used to inoculate batches of tea.

2.7. Preparation of inoculated tea samples for HPLC analysis

Green and black tea infusions were used for the experiments. Aqueous extracts were made by adding 2 g of tea leaves to 100 ml of boiling water and allowing the suspension to stand for 10 min. The infusion was then filtered through cotton to eliminate rough particles, and then sterile filtered through a 0.45-μm membrane. The infusions obtained were approximately the strength of a "normal cup of tea" (Yam et al., 1997).

Four batches (10 ml) of green or black teas were prepared as follows: two batches only contain black tea or green tea infusions and served as control. The two other batches were inoculated with a mixture (about 10^8 UFC/ml) of *L. bulgaricus* and *S. thermophillus*, resuspended in green or black teas. All batches were then incubated at 37 °C. After 48 h of incubation, all aqueous samples were harvested by centrifugation at 6000 rpm for 10 min, green and black tea extracts for HPLC analysis were then prepared.

2.8. Extraction of tea catechins for HPLC analysis

Tea infusion samples of 10 ml volume were extracted three times with 3×10 ml ethyl acetate at room temperature.

The ethyl acetate fractions were combined and subsequently dried over anhydrous sodium sulphate and then evaporated to dryness using a rotary evaporator at 40 °C. The residue was immediately re-dissolved in a final volume of 1.5 ml of methanol.

2.9. HPLC Instrumentation

The HPLC equipment (Agilent Technologies, Santa Clara, California, USA) consisted of an Agilent 1200 series quaternary pump with vacuum degasser, an Agilent 1200 series auto-injector, and an Agilent 1200 series diode array detector. Chromatograms were recorded at 280 nm with spectra values (180–400 nm) taken continuously throughout the elution. Data collection and subsequent processing were performed using HP Chem-Station software.

2.10. HPLC conditions

The analytical column was an Eclipse XDB-C18 (150 mm × 2.1 mm i.d., 3.5 μm; Agilent Technologies, USA) and operated at room temperature. The method of Zuo et al. (2002) was used with slight modifications. The mobile phase A was water–acetic acid (97/3, v/v) and the mobile phase B was methanol. The chromatographic program run consisted of an isocratic step of 100% solvent A for 1 min, followed by a 100-to-37% solvent A linear gradient step for 27 min, then a 37-to-100% solvent A linear gradient step for 2 min and finally an isocratic step of 100% solvent A for 10 min. The flow rate was set at 0.1 ml/min.

3. Results and discussion

Various additives of nondairy origin are used in the manufacture of milk products because of their beneficial contribution to the sensory, therapeutic, or other properties of dairy foods (Varga & Szigeti, 1998). However, some of these substances may contain spoilage microorganisms, thereby negatively affecting the shelf life of finished products.

In the present study, tea leaves were added to the pasteurised milk immediately after ebullition in order to let the tea infuse into the milk and thus release its active components to protect the final product against pathogenic bacteria. Nevertheless, a hygienic level of the final product was tested in this work. The colony counts were in conformity with food regulations and remained so throughout all experiments. Yeasts, sulphite reducing *clostridia* and *Salmonella* spp. were not detected during the storage period. These results are in concordance with the literature emphasising the antimicrobial activities of tea against many pathogenic bacteria (Chou et al., 1999; Hamilton-Miller, 1995; Jaziri & Hamdi, 2005; Yam et al., 1997).

Chromatographic studies were used to evaluate the effect of *S. thermosphilus* and *L. bulgaricus* yogurt bacteria on six phenolic compounds (CG), (EGC), (C), (EGCG), (GCG) and (ECG) of green and black teas. The calibration curves (Fig. 2) were determined with working mixed standard solutions prepared in methanol and containing a mixture of the six phenolic compound standards. The injection of each mixed standard solution was done in triplicate. The results obtained showed good correlation ($R^2 = 0.999$; 0.999; 0.998; 0.985; 0.999 and 0.951, respectively for CG, ECG,

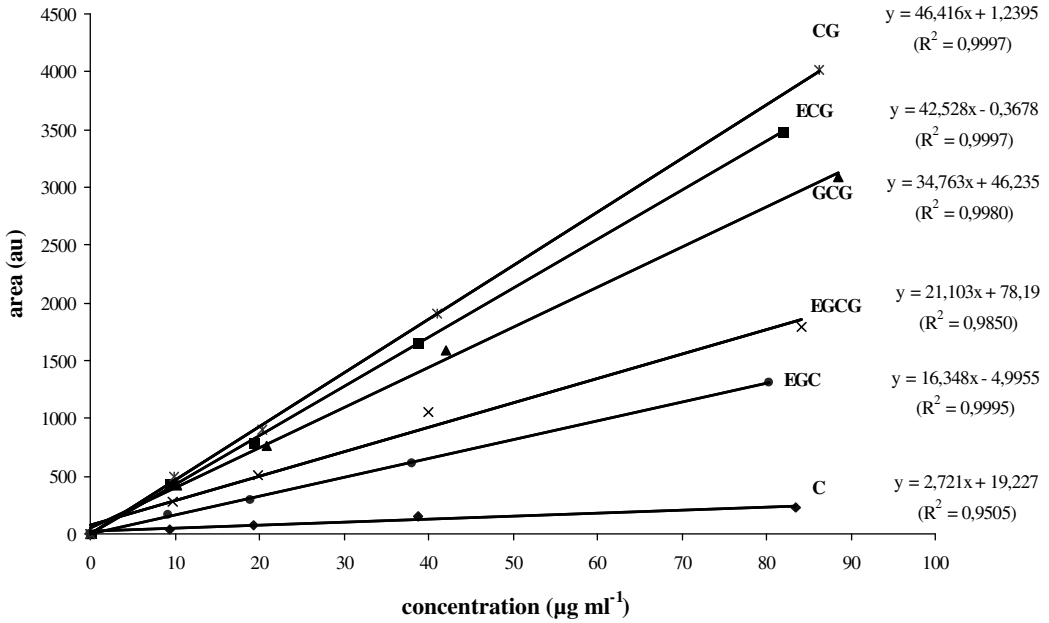

Fig. 2. Calibration curves of (EGC), (CG), (C), (EGCG), (GCG) and (ECG).

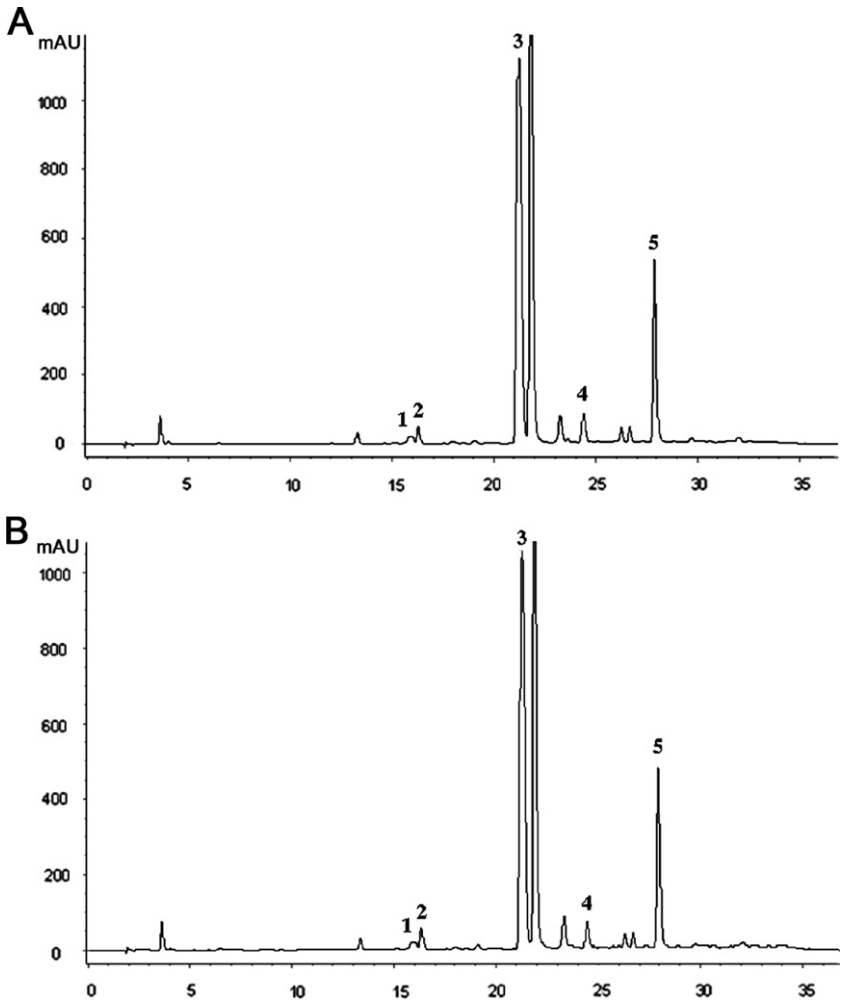

Fig. 3. HPLC chromatogram of phenolic compounds (catechins) in (A) green tea and (B) Inoculated green tea. Peaks correspond to: (1) EGC: epigallocatechin; (2) C: catechin, (3) EGCG: epigallocatechingallate; (4) GCG: gallocatechingallate; (5) ECG: epicatechin gallate.

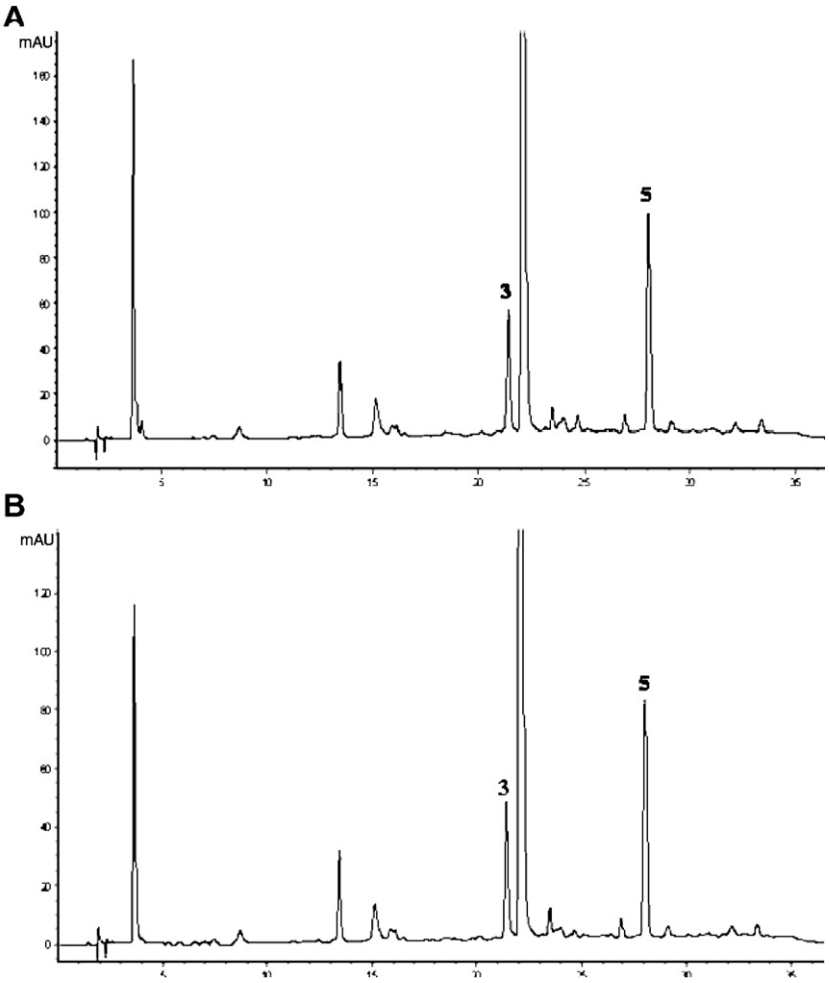

Fig. 4. HPLC chromatogram of phenolic compounds (catechins) in (A) Black tea and (B) Inoculated Black tea. Peaks correspond to: (3) EGCG: epigallocatechingallate; (5) ECG: epicatechin gallate.

GCG, EGCG, EGC and C) for the different standards and the range of linearity was wide.

Figs. 3A and 4A show the chromatographic profiles of green and black tea samples, respectively, before the treatment with *S. thermosphilus* and *L. bulgaricus* yogurt bacteria. The chromatograms showed good separation of the six phenolic compounds allowing their identification and quantification easily with a predominance of GCG and EC.

After the treatment of the same samples with the yogurt bacteria, the chromatograms obtained showed no significant modification ($P < 0.05$) of the phenolic compounds of both green and black tea samples (Figs. 3B and 4B. Table 1 gave the composition of phenolic compounds in inoculated and non inoculated tea samples. The results obtained give a clear confirmation that the yogurt bacteria did not affect significantly ($P < 0.05$) the composition of the phenolic compounds of green and black teas.

Before the beginning of production and storage trials, preliminary studies were conducted to see the effect of tea addition to milk on acidity during fermentation. The acidity of milks dropped as expected over the 6 h period of incubation and no significant

Table 1
Composition of phenolic compounds (μg/ml)[a] in green and black tea samples inoculated or not with yogurt bacteria ($n = 3$)

Phenolic compounds	Green tea		Black tea	
	Control	Inoculated	Control	Inoculated
EGC	8.15 ± 0.03	7.04 ± 0.05	ND	ND
C	154.33 ± 0.05	183.42 ± 0.04	ND	ND
EGCG	850.21 ± 0.04	782.87 ± 0.04	24.80 ± 0.02	19.98 ± 0.03
GCG	24.66 ± 0.05	20.88 ± 0.05	ND	ND
ECG	135.54 ± 0.04	120.35 ± 0.03	23.31 ± 0.03	19.89 ± 0.02
CG	ND	ND	ND	ND

ND: not detected.
[a] Values are mean ± SD phenolic compound concentrations ($n = 3$).

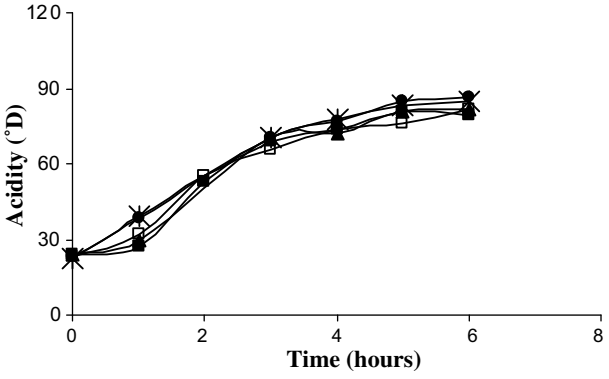

Fig. 5. Acidity evolution of the yogurt during fermentation. □: Control; ■: 2% (w/v) green tea; ●: 4% (w/v) green tea; ▲: 2% (w/v) black tea; x: 4% (w/v) black tea.

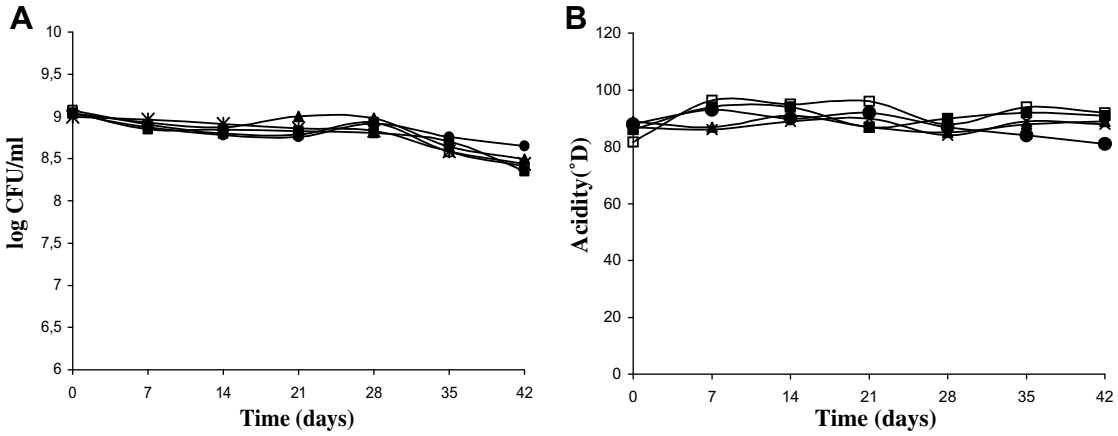

Fig. 6. (A) Yogurt bacteria survival and (B) acidity evolution, during 42 days of refrigerated storage. □: Control; ■: 2% (w/v) green tea; ●: 4% (w/v) green tea; ▲: 2% (w/v) black tea and x: 4% (w/v) black tea.

($P < 0.05$) differences (Fig. 5) in acidity were observed among the treatments, indicating that tea neither supported nor impeded lactic acid production by *S. thermophilus* and *L. bulgaricus* at this stage.

Thereafter, viability of the starter organisms in control and tea yogurts during 42 days of refrigerated storage at 4 °C was monitored weekly. The addition of tea to milk before yogurt fermentation did not significantly ($P \leqslant 0.05$) influence the growth and survival of both streptococci and lactobacilli during production and subsequent refrigerated storage of yogurts (Fig. 6A). The yogurt microflora was found to be present at sufficiently high levels both at the beginning and the end of the six-week storage period. Legislation in France and many other countries require the presence of at least 10^7 yogurt bacteria per gram of yogurt product at the time of consumption (Anonymous., 2001). Ranging between 8.28 and 9 log CFU/ml, all the counts of streptococci and lactobacilli largely exceeded this value throughout the entire storage period.

As a result of these mentioned observations, all the five experimental yogurt formulations fulfilled the legal requirements in terms of acidity and levels of viable starter organisms during the whole storage period, with no significant ($P < 0.05$) differences among the various treatments (Fig. 6A and B).

These findings indicate that yogurt lactic acid bacteria were not inhibited during their growth or during their survival by different types and concentrations of tea. These results are in concordance with recent studies (Lee, Jenner, Lowa, & Lee, 2006) showing that some lactic acid bacteria such as *Lactobacillus* spp. were not severely affected by tea phenolic compounds in opposition to other pathogenic bacteria.

According to previous studies, McCue Patrick and Shetty (2005) demonstrated that phenolic antioxidants were not affected by yogurt microflora during yogurt production from soymilk in presence of kefir culture. In another study, Kachouri and Hamdi (2006), mentioned that some lactic acid bacteria possessed significant antioxidative activity, allowing the preservation of catechins from oxidation during yogurt fermentation. Therefore, tea phenolic compounds in yogurt products will be able to keep their antimicrobial and antioxidant activities and could even constitute novel natural colorants and be considered as "functional" (Coisson, Travaglia, Piana, Capasso, & Arlorio, 2005). Moreover, Leenen et al. (2000) and Van het Hof, Kivits, Weststrate, and Tijburg (1998), have also shown that adding milk to teas did not affect the absorption of tea catechins and antioxidant capacity in human plasma. All these results give more interest to the addition of teas or tea phenolic compounds to milk or dairy products like yogurts to insure high health benefits and protection against spoilage microorganisms.

4. Conclusion

The present work is considered as the first report demonstrating that the presence of green and black teas at 2.0% to 4.0% (w/v) did not influence the fermentation of yogurts and the survival of characteristic microorganisms in yogurts during a six-week storage period at 4 °C. Similarly, green and black teas have no effect on the pH and the lactic acid levels of final products.

Therefore, addition of green or black teas in the process milk used for making yogurts is recommended because tea is a natural herbal product with a wide range of beneficial and nutritional properties; this makes this new yogurt a functional food. Furthermore, at a concentration of 2.0% (w/v), which is approximately the strength of a common cup of tea, this may improve the taste of yogurts without having an inhibitory effect on the starter bacteria.

Acknowledgements

The authors would like to thank Amira Anène and Inès Mahouachi from the National Institute for Research and Physical–chemical Analysis (INRAP) for their help in the HPLC analysis.

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