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THE SUPPLEMENTARY EFFECT OF BLACK AND GREEN TEA INFUSION ON ANTIMICROBIAL ACTIVITIES OF KEFIR

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ABSTRACT

The influence of supplementation with green and black tea on microbiological properties and antimicrobial activities of kefir was investigated during 21 days of storage. The samples supplemented with 2% either green or black tea had higher viable counts of both kefir cultures than those of supplemented with the ratio of 4%. Both green and black tea extracts showed antimicrobial activity on *Escherichia coli, Bacillus cereus, Staphylococcus aureus, Candida albicans* however this effect was detected higher in samples containing green tea.

Keywords: Green tea, Black tea, Kefir, Antimicrobial, Viability

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Introduction

Tea (*Camellia sinensis*, family Theaceae) is commonly consumed worldwide having various health benefits and physiological functionalities, such as antioxidative, anticarcinogenic and antimicrobial effects (Michalczyk & Zawiślak 2008: Chan et al 2011; Archana & Abraham 2011). The most important bioactive substances responsible for these health effects present in tea are tea polyphenols. Tea catechins are the major components of polyphenols, which consist of (-)-epigallocatechin gallate (ECg), (-)-epicatechin (EC), and their epimerization isomers (+)-gallocatechin gallate (GCg), (+)-gallocatechin (GC), (+)-catechin gallate (Cg), and (+)-catechin (C) (Goto et al 1996). The antimicrobial activity of tea which inhibit many undesired microbial growth are mainly related to their polyphenolic components (Michalczyk & Zawiślak 2008). The extracts of *Camellia sinensis* have been determined to inhibit the growth of *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Candida albicans* and *Bacillus cereus* in many studies (Chan et al 2011; Archana & Abraham 2011; Kumar et al 2012; Inamdar et al 2014).

In recent years, different ingredients have been used to improve the therapeutic benefits and some functional properties of kefir. Green and black teas were used because of their benefits to human health and their popular consumption worldwide in some dairy products such as milk, yoghurt, fermented milk and some other probiotic dairy products (Jaziri et al 2009; Najgebauer-Lejko et al 2011; Marhamatizadeh et al 2013; Ye et al 2013; Najgebauer-Lejko 2014; Ma et al 2015).

The objective of this study was to investigate the viability of kefir microorganisms, antimicrobial properties in presence of two different ratios (2% or 4%) green and black teas during refrigerated storage.

Materials and Methods

Material

UHT cow's milk used in the studies obtained from Pinar Sut Co. (Izmir, TURKEY). Commercial freeze-dried kefir starter culture containing *Lactococcus lactis* spp. *lactis*, *Lactococcus lactis* spp. *cremoris*, *Lactococcus lactis* spp. *diacetylactis*, *Leuconostoc mesenteroides* spp. *cremoris*, *Lactobacillus kefyr*, *Kluyveromyces marxianus*, and *Saccharomyces unisporus* spp. obtained from Danisco DC – Kefir (Olsztyn, Poland). Green tea (Kardelen) and black tea (Caykur 1. Nevi) leaves were purchased from Caykur Co. (Rize, Turkey). Foodborne pathogens and spoilage microorganisms (*Escherichia coli* CECT 4267, *Bacillus cereus* CECT 131, *Staphylococcus aureus* ATCC 12600, *Candida albicans* ATCC14053*)* for antibacterial activity were obtained from the collection Department of Dairy Technology (Ege University, Izmir, Turkey).

Methods

Production of Kefir: The freeze dried kefir culture was propagated by inoculating in skim milk which was heated at 90 °C for 30 min before the inoculation. The inoculated milk was incubated at 25°C until pH 4.6 was reached, then stored overnight at 4°C in refrigerator. The whole milk was heated to 85 °C and waited for 10 min, then fortified with green or black tea at levels of 2% and 4% (w/v). The teas were infused for 10 min then different batches were filtered through sterile cotton to remove the particles. The milk samples were ten cooled to 25 °C and inoculated 3% kefir culture and divided into 200 mL plastic containers and incubated at 25 °C until pH 4.6 was reached. Following the fermentation, the samples were cooled and stored at 4 °C for 21 days for the analyses. Five different beverages were produced: CK: control kefir, 2BK: kefir supplemented with 2% black tea, 4BK: kefir supplemented with 4% black tea, 2GK: kefir supplemented with 2% green tea, 4GK: kefir supplemented with 4% green tea.

pH analyses: The pH was determined with a pH meter (Hanna pH 211 Microprocessor, Portugal).

Microbiological analyses: Lactobacilli counts in kefir samples were enumerated in MRS agar (pH 5.8) (Merck/1.10660, Darmstadt, Germany) via anaerobic incubation at 42°C for 48 h; whereas Lactococci in the kefir samples were counted in M17 agar (pH 6.9) via aerobic incubation at 37°C for 48 h. Yeasts were enumerated using YGC Agar (pH 6.8) via incibation at 25°C for 72 h (Merck Kga A, Darmstadt, Germany) and incubated (Bracquart, 1981).

Antimicrobial activity: Antibacterial susceptibility testing was done by using disc diffusion method (Radji et al 2013). To check antimicrobial activity of samples, sterile tripton soy agar plates were used. Tripton soy agar was prepared and autoclaving at 121°C for 15 minutes. The medium was poured in sterile petri plates under aseptic conditions. Then allowed the media to solidify at room temperature and stored at 4°C until use. After solidification, 0.2 ml of inoculum suspension was inoculated with micropipette and spread uniformly with sterile glass spreader over agar surface, the inoculum was allowed to dry for 5 minutes. 50µl concentration of samples was loaded on sterile individual discs. The loaded discs were placed on the surface of medium and the sample was allowed to diffuse at least for 5 minutes. The plates were kept for incubation at 37°C for 24-48 h. Methanol, ethanol and distilled water were used as

negative control. Plates were observed after 24-48 h incubation for appearance of zones of inhibition around the discs. Antimicrobial activity was evaluated by measuring diameter of zones of inhibition (in millimeters) of microbial growth.

Statistical Analysis: The experiments were performed in twice with three parallel. Six values for each sample were averaged (n=6). The data obtained was processed by one-way ANOVA using the general linear model procedure of the SPSS version 11.05 (SPSS Inc., Chicago, IL, USA). The means were compared with the Duncan test at $p<0.05$ level.

Results and Discussion

Changes in pH values

pH values of kefir samples produced from milk fortified with black and green tea infusion at different ratios and the changes in these values during storage are given in Figure 1. pH value of the samples varied between 4.38 and 4.66. The changes in pH values of our samples in terms of storage and black and green tea fortification ratios were statistically significant ($p<0.05$). These results are similar to those obtained in other studies (Irigoyen et al. 2005; Fontan et al. 2006). It was reported that the quality of the milk, dry matter content, the diversity of microorganisms that constitute the kefir culture, kefir production technologies, fermentation temperature, fermentation duration and the time from production to consumption were effective on the composition of kefir (Güzel-Seydim et al. 2005). Najgebauer-Lejko (2014), in their study on bioyogurt and acidophilus milk fortified with green tea infusion at different ratios, reported that the pH values of biyogurts and acidophilus milks were slightly higher in samples with higher levels of green tea supplementation.

Microbiological Properties

The changes in *Lactococcus* spp. and *Lactobacillus* spp. and yeast counts of kefir samples containing green and black tea extracts during storage are given in Table 1. *Lactococcus* spp. counts of kefir samples produced by blending with 2% and 4% green and black tea extracts varied between 8.87 log cfu/mL and 8.72 log cfu/mL on the 1st day of the storage period. Although there were no significant differences between the samples on the 1st day, *Lactococcus* spp. counts in the samples containing tea extracts were slightly lower. There were significant decreases in *Lactococcus* spp. counts related to both the storage period and the rate of green/black tea extract fortification ratios. *Lactococcus* spp. counts determined in kefir samples at the 21st day of the storage varied between 6.44 log cfu/mL and 6.76 log cfu/mL.

Lactobacillus spp. counts of kefir samples ranged between 8.71 log cfu/mL and 8.86 log cfu/mL on the 1st day of storage whereas the values changed between 6.31 log cfu/mL and 6.70 log cfu/mL on the 21st day of storage. In the evaluation of all the results, although there were no significant differences between *Lactococcus* spp. and *Lactobacillus* spp. counts found in the traditional kefir culture, there were substantial decreases related to the storage period and the ratio of tea extracts.

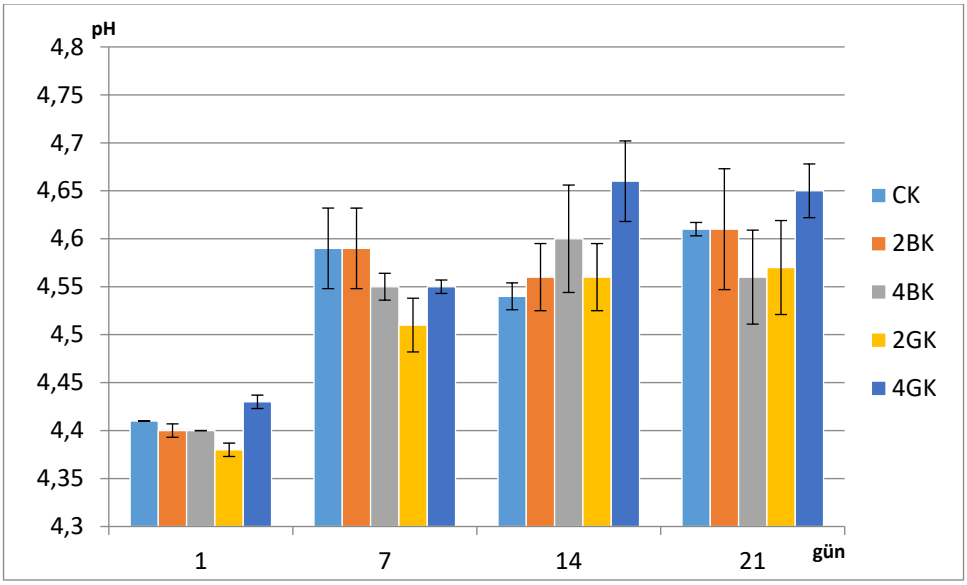

Figure 1. Changes in pH during 21 days of storage in kefir samples.

Table 1. The changes in *Lactobacillus* spp., *Lactococcus* spp. and yeast counts of kefir samples during storage

	DAY	CK	2BK	4BK	2GK	4GK
Yeast	1	5.53±0,08^{B}	5.59±0.03^{A}	5.59±0.02^{A}	5.55±0.03^{A}	5.54±0.02^{A}
	7	5.47±0.03^{B}	5.43±0.03^{A}	5.33±0.03^{A}	5.34±0.06^{A}	5.29±0.06^{B}
	14	5.39±0.04^{aB}	4.54±0.07^{cB}	4.94±0.02^{bB}	4.52±0.04^{cB}	4.36±0.02^{dC}
	21	6.56±0.04^{aA}	3.81±0.01^{bC}	3.69±0.00^{bcC}	3.77±0.01^{bC}	3.63±0.00^{cD}
Lactobacilus spp.	1	8.86±0.01^{A}	8.78±0.01^{A}	8.74±0.02^{A}	8.77±0.01^{A}	8.71±0.05^{A}
	7	8.63±0.01^{A}	8.58±0.02^{A}	8.42±0.01^{A}	8.46±0.10^{A}	8.31±0.09^{A}
	14	7.73±0.08^{B}	7.67±0.05^{B}	7.62±0.01^{B}	7.63±0.02^{B}	7.56±0.02^{B}
	21	6.70±0.07C	6.51±0.01^{C}	6.38±0.05^{C}	6.48±0.03^{B}	6.31±0.01^{C}
Lactococcus spp.	1	8.87±0.04^{A}	8.87±0.01^{A}	8.75±0.08^{A}	8.84±0.01^{A}	8.72±0.04^{A}
	7	8.52±0.02^{A}	8.48±0.04^{A}	8.40±0.02^{A}	8.46±0.09^{A}	8.35±0.07^{A}
	14	7.67±0.01^{B}	7.63±0.03^{B}	7.51±0.01^{B}	7.60±0.12^{B}	7.48±0.06^{B}
	21	6.76±0.05^{C}	6.66±0.01^{C}	6.51±0.07^{C}	6.59±0.01^{C}	6.44±0.10^{C}

$^{a-d}$ Means ± standard deviations in the same row with different superscript lowercase letters are significantly different ($p<0.05$).
$^{A-D}$ Means ± standard deviations in the same column with different superscript uppercase letters are significantly different ($p<0.05$).

Evaluating all the values obtained in kefir samples, *Lactococcus* spp. and *Lactobacillus* spp. have a symbiotic relation, and first *Lactococcus* spp. is active during fermentation and subsequently *Lactobacillus* spp. shows activity. While the baseline *Lactococcus* spp. and *Lactobacillus* spp. counts were maintained at the first 7 days of storage, the counts began to decline on the 14th day and were found to be statistically significant. However, the differences between the *Lactococcus* spp. and *Lactobacillus* spp. counts in the control sample, 2% and 4% green and black tea samples were statistically insignificant ($p> 0.05$). This shows that 2% and 4% green and black tea additions do not affect the bacteria in kefir production.

Najgebauer-Lejko (2014) investigated the effect of using green tea extracts on the microbiological properties of bioyoghurt and acidophilus milk and found similar viability with our study. The authors determined Lactobacilli counts between 7.21 log cfu/g and 8.29 log cfu/g in bioyoghurt samples whereas the values were found between 8.72 log cfu/g and 9.02 log cfu/g in acidophilus milk samples. On the other hand, Bifidobacteria counts changed between 6.66 log cfu/g and 7.54 log cfu/g in bioyoghurt samples. The researchers indicated that the interaction between the ratio of tea extracts and the bacteria species had a significant effect on the viability. This contrariness with our study about the effect of addition of tea on bacterial counts can be due to the different types of fermented dairy product used in the studies.

In another study, Marhamatizadeh et al. (2013) investigated some properties of probiotic yoghurt, containing *L. acidophilus* and *B. bifidum*, fortified with green tea extract. The viable counts of *L. acidophilus* and *B. bifidum* in sterile low fat milks fortified with 0.3%, 0.6% and 0.9% green tea extracts were found to be higher than that of control sample. The addition rate of green tea extract significantly affected the viability of *L. acidophilus* while the highest counts were determined on 14th day of storage.

Yeasts are important in kefir fermentation because of the production of ethanol and carbon dioxide, which give the kefir drink its unique taste. The counts of yeasts did not generally alter significantly in all kefir samples during two weeks of storage ($p>0.05$) whereas the values statistically decreased in tea extract supplemented samples on 14th and 21st days of storage ($p<0.05$). Statistically significant differences were found between control sample and the samples supplemented with black or green tea in terms of yeast count ($p <0.05$) on 14th and 21st days of storage. Black and green tea supplemented kefir samples had lower yeast counts than that of control sample during the last two weeks of storage probably due to the suppression effect of tea on yeast viability. The yeast viability significantly decreased ($p<0.05$) when the ratio of green tea increased from 2% to 4% on the reported days. The yeast population level in our study were found to be lower than the enumerations reported by Wszolek et al. (2005), Witthuhn et al. (2005) and Irigoyen et al. (2005).

Consequently, it is possible to say that microorganism variety found in the fermented dairy product, the addition rate of tea extracts and also the type of tea used in the manufacture can affect the microbiological characteristics of the product. Moreover, incubation conditions, storage period, level of unwanted microflora and their enzymes, concentration of nutrients in the medium and the different phenolic compounds found in black or green tea might influence the viability of starter cultures (O'Connel & Fox, 2001; Marhamatizadeh et al., 2009; 2013).

Antimicrobial Activity

In the study, the antimicrobial effects of kefir samples containing 2% and 4% green and black tea extracts were determined on *Echerichia coli*, *Bacillus cereus*, *Staphylococcus aureus* pathogenic bacteria and *Candida albicans* which is a pathogenic yeast. Antimicrobial effect was determined by measuring zone diameters formed as a result of antimicrobial effect by disc diffusion method in 50 µL kefir samples containing 2% - 4% green and black tea extracts. It was found that tea extracts had antimicrobial effects on the mentioned microorganisms at both ratios and this effect was even higher in samples containing green tea extracts. Zone diameters formed by the tea extracts as a result of antimicrobial effects are shown in Table 2. In Table 2, it is seen that the highest antimicrobial effect by 2% and 4% green tea extracts were on *S. aureus.* Zone diameters varied between 12.77 mm and 9.45 mm in 2% green tea extract use and between 16.32 mm and 10.25 mm in 4% green tea extract use. Green tea extracts had a similar effect on other microorganisms and antimicrobial activity on the microorganisms from highest to lowest was sorted *B. cereus, C. albicans* and *E.coli*, respectively. Additionally, antimicrobial activity showed a decreasing course during storage. At the 1st day of the storage, zone diameter formed in *E. coli* in kefir samples containing 2% green tea was 9.37 mm while it was 11.15 mm in *B. cereus* and 16.35 mm in *C. albicans*. These values decreased to 6.65, 7.25 and 11.50 mm respectively at the end of the 21st day. Using 4% green tea extract significantly increased the antimicrobial activity especially at the 1st day and the zone diameters were 15.32 mm for *E. coli*, 14.50 mm for *B. cereus* and 23.52 mm for *C. albicans*. Antimicrobial activity decreased at the 21st day and the inhibitions zoned were measured to be 10.55 mm for *E. coli*, 9.65 mm for *B. cereus* and 15.32 mm for *C. albicans*.

Similar results were observed in black tea extract use, however the antimicrobial effect was lower. At the 21st day, the inhibition zones determined in kefir samples containing 2% black tea were 6.20 mm for *E. coli*, 6.87 mm for *B. cereus*, 6.65 mm for *S. aureus* and 8.15 mm for *C. albicans.* Unlike green tea extracts, the antimicrobial effect of black tea extracts on *C. albicans* was higher. Similar to those of containing green tea, significantly higher antimicrobial activity values were determined in kefir samples containing 4% black tea extracts at the 1st day, however decreased in the further days. The antimicrobial activity determined at the 21st day of the storage was 7.47 mm for *E.coli*, 6.40 mm for *B. cereus*, 8.15 mm for *S. aureus*, and 11.05 mm for *C. albicans.*

Water extracts of the tea leaves are consumed for centuries. Examining the physicochemical properties of tea leaves, it was determined that they contain alkaloids, saponins, tannins, catechins and polyphenols and tea leaves are used against microorganisms for their antimicrobial activity. The main difference between green and black tea leaves is the fermentation stage and the leaves of black tea are fermented, oxidized and then dried. However, phytochemicals found in the composition of tea are very sensitive to the oxidation stage. The studies showed that green tea extracts inhibited *S. aureus, Vibrio parahaemolyticus, Clostridum perfringens, Bacillus cereus* and their strains. Although green tea extracts contain 30-40% water soluble polyphenols, this level decreases to 3-10% in black tea extracts. According to the studies, epigallocatechin gallate, epicatechin gallate, epigallo catechin, epicatechin are considered as the most important antioxidant components and it was determined that the most important effects of these components are on entero pathogens (Archana & Abraham, 2011; Diane et al., 2007). Archana & Abraham (2011), in their study, reported that *E. coli, Enterococccus faecalis, S. aureus, Pseudomonas aeruginosa* and *C. albicans* are very sensitive to fresh green tea extracts, however the antimicrobial activity decreased in commercial green tea. Antimicrobial activity in black tea was very low and, furthermore, it had no effect on *E. faecalis, P. aeruginosa* and *C. albicans*. Katsuhiro et al. (1999) determined that green tea extracts have antibacterial effects on *Helicobacter pylori* and reported that it has a positive effect in the treatment of gastrointestinal problems related to this bacterium.

Table 2: Antimicrobial activity of kefir samples during storage given as the diameter of inhibited zone (mm)

	DAY	CK	2BK	4BK	2GK	4GK
	1	6.35±0.02	7.90±0.26	11.00±0.36	9.37±0.26	15.32±0.70
	7	6.00±0.08	7.77±0.38	9.87±0.09	9.32±0.22	14.52±0.93
E.coli	14	5.17±0.17	7.15±0.10	8.52±0.12	7.57±0.05	12.15±0.35
	21	3.35±0.26	6.20±0.16	7.47±0.10	6.65±0.17	10.55±0.06
	1	7.50±0.11	9.55±0.10	10.85±0.06	11.15±0.19	14.50±0.34
	7	7.75±0.29	8.92±0.09	9.60±0.37	10.67±0.15	13.85±0.05
B. cereus	14	5.50±0.08	7.30±0.24	7.52±0.09	8.67±0.21	11.60±0.08
	21	4.35±0.30	6.87±0.09	6.40±0.28	7.25±0.29	9.65±0.10
	1	8.15±0.19	10.40±0.16	12.65±0.24	12.77±0.33	16.32±0.21
	7	5.92±0.15	9.72±0.09	11.60±0.14	12.55±0.05	15.25±0.13
S.aureus	14	4.27±0.32	8.07±0.09	9.62±0.05	10.77±0.30	11.35±0.27
	21	2.65±0.09	6.65±0.13	8.15±0.10	9.45±0.19	10.25±0.46
	1	10.13±0.26	12.47±0.09	18.50±0.11	16.35±0.25	23.52±0.15
	7	9.72±0.15	11.30±0.32	16.22±0.26	15.60±0.18	21.57±0.05
C.albicans	14	8.37±0.26	9.57±0.05	13.50±0.26	13.30±0.11	18.57±0.24
	21	7.40±0.16	8.15±0.10	11.05±0.37	11.50±0.26	15.32±0.09

Wu et al. (2007) reported that water extracts of various tea types including green tea showed an antimicrobial activity against *S. aureus* and *B. subtilis* at 2 mg/mL concentration, however no antimicrobial effect was observed on Gram (-) *E. coli*. On the other hand, it was stated that the level of resistance of Gram (-) bacteria against the extracts were related to the lipopolysaccharides in the cell membrane and the antimicrobial activity was higher in fresh tea leaves due to their high polyphenol content (Alzoreky & Nakahara, 2003; Negi et al., 2005; Chou et al., 1999). Kumar et al. (2012) investigated the antibacterial activity of green tea leaves against environment-originated *S. aureus*, *Streptococcus* ssp., *Pseudomonas aeruginosa*, *Bacillus* ssp., *E.coli* and *Proteus* species with disc diffusion method. Antibacterial activity was tested at 10 UI, 20 UI and 30 UI extract concentrations, significant levels of antibacterial activity was observed in all concentrations and the inhibition zone diameters varied between 7 and 13 mm. Chou et al. (1999), in their study, investigated the antimicrobial activity of different tea types against *Bacillus subtilis, E.coli, Proteus vulgaris, Pseudomonas fluorescens, Salmonella* spp. *and S. aureus.* Among the six bacteria species, the most sensitive bacterium to the extracts was *P. fluorescens* whereas the most resistant bacterium was *B. subtilis*. *E.coli, S. aureus, P. vulgaris* and *Salmonella* spp. were inhibited at similar ratios and the activity decreased in all species when fermented tea extracts were used. Chan et al. (2011) investigated the antioxidant and antimicrobial activities of green, black and some herbal tea extracts. In the study, antimicrobial activity of tea extracts was investigated against Gram (+) *Micrococcus luteus, S. aureus* and *B. cereus* and Gram (-) *E. coli*, *Salmonella typhi* and *P. auregonisa* using disc diffusion method. The study showed that all extracts were effective on Gram (+) bacteria whereas they had no effect on Gram (-) bacteria. The highest antimicrobial activity in green tea extracts was against *M. luteus* and *B. cereus*, whereas the lowest was against *S. aureus.* Although black tea extracts showed a similar antimicrobial activity, they had no effect on *S. aureus*. The researchers also stated that lipoproteins and lipopolysaccharides found in the cell membranes of Gram (-) bacteria increased the resistance against antimicrobial agents.

Abd-Allah et al. (2011), in their study, investigated the antimicrobial effects of black tea and milk beverages containing black tea on *Steptococcus mutans* and *Lactobacillus* sp. found in the oral flora. The analys results of samples obtained from children showed that black tea and milk beverages containing black tea had a highly significant bacterial counts reduction against these cariogenic bacteria by different rates (43.6% - 83.3%). Inamdar et al. (2014), in their study, determined that water extracts of tea leaved had a strong antifungal effect on *Sacchromyces cerevisiae* and

low antifungal effect on *Candida albicans*, whereas no antifungal effect was observed on *C. tropicals*. It was determined that the antifungal effects of alcohol extracts of teas were very high on *C. albicans* and *cerevisiae* whereas they were very low on *C. tropicalis*. It was observed that the results obtained in many studies (Archanda & Abraham, 2011; Chou et al 1999; Erol et al. 2009; Katsuhiro et al. 1999; Kumar et al. 2012; Mandal et al. 2011; Radji et al. 2013; Shetty et al. 1994) support the results obtained in the present study and varied depending on the extraction type, type of tea and the properties of microorganisms.

Conclusions

Lactobacillus ssp. and *Lactococcus* ssp. counts of kefir samples produced with 2% and 4% green and black tea was above 10^6 - 10^7 cfu / mL throughout of storage. It appears that the green and black tea addition did not adversely affect the pH values and the samples retained their probiotic properties. On the other hand, green tea improved the antimicrobial activities of kefir higher compared to black tea. This effect was stronger when the supplementation ratio increased from 2% to 4%. Therefore, fortification of kefir with green tea can be an alternative pathway to create a functional dairy product having both nutritional and health benefits.

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