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Increased Preservative and Antimutagenic Activities of *Kimchi* with Addition of Green Tea Leaves

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Abstract

Preservative and antimutagenic effects of green tea leaves added Chinese cabbage *kimchi* (GK1, GK2, GK3, and GK4 : 1, 2, 3 and 4 of green tea leaves (GTL) in proportion to 100 of salted Chinese cabbage were added to *kimchi*) were compared to those of the Chinese cabbage *kimchi* without GTL (control *kimchi*, CK). Fermentation period of GKs was further delayed than that of CK. The initial pH and acidity between GKs and CK were similar, but the time to reach optimally ripened status of *kimchi* (pH 4.3) was different. CK took 6 days, while GK1, GK2, GK3 and GK4 took 6, 10, 12 and 14 days at 10°C, respectively. The growth of *Leuconostoc* sp. and *Lactobacilus* sp. in GKs delayed comparing to those in CK. Among GKs, as the added amount of green tea leaves increased, the growth of lactic acid bacteria was retarded. The antimutagenic effects of juices from GKs and CK were studied against aflatoxin B_1 (AFB_1) in the Ames test on *Salmonella typhimurium* TA100 and N-methyl-N′-nitro-N-nitroso-guanidine (MNNG) in the SOS chromotest using *E. coli* PQ37. Juices from optimally ripened GKs (pH 4.3) showed 52~76% inhibition rates against the indirect mutagen, aflatoxin B_1 induced mutagenicity while 49% inhibition rate by CK in the Ames test. Juices from GKs and CK showed 44~67% and 36% inhibition rate against direct mutagen, MNNG (70 ng/assay) induced mutagenicity in the SOS chromotest. Thus GKs delayed fermentation period of *kimchi* and exhibited higher antimutagenic activity than CK.

Key words: green tea leaves, *kimchi*, aflatoxin B_1, MNNG, antimutagenicity

INTRODUCTION

Kimchi is a major Korean traditional fermented food, as a major source of vitamins, minerals and dietary fiber, which is prepared with various vegetables, condiments, etc. There are many types of *kimchi* depending on raw ingredient and preparation method used (1). Because of some antioxidatative nutrients and phytochemicals in *kimchi* such as vitamin C, β-carotene, minerals, dietary fiber, β-sitosterol, etc., optimally ripened *kimchi* shows inhibitory effects on the growth of cancer cells (1,2).

Recent investigations in several laboratories have found that some polyphenolic compounds isolated from green tea have anticarcinogenic activity both *in vitro* and *in vivo* (3,4). The major green tea leaf polyphenols are (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG), and (-)-epigallo-catechin-3-gallate (EGCG). There are also reports that the polyphenols from green tea can show antioxidative activity (5), antimutagenicity (6), antitumorgenicity and anticarcinogenicity (7). Epidemiologicical studies have not provided conclusive results but tend to suggest that green tea may reduce the risk associated with cancers of bladder (8), prostate (9), esophagus (10) and stomach (11). Experiments on a molecular level have shown that green tea caused the cancer cell cycle arrest and induced apoptosis (12). The antimicrobial capacity (13) and the multiple inhibitory effects of green tea on mutagenicity (3-14) led us to study whether the addition of green tea leaves to the *kimchi* may delay of the fermentation period and increase on antimutagenicity of the *kimchi*.

To evaluate the possibility of the use of green tea leaves as a subingredient of *kimchi*, the changes in pH, acidity and the level of lactic acid bacteria of the various amounts of green tea leaves added Chinese cabbage *kimchi* were investigated. The antimutagenic effects of juices from the green tea leaves added *kimchi* (GKs) were compared to those of the control *kimchi* (CK) in the Ames test and in the SOS chromotest systems.

MATERIALS AND METHODS

Preparation of *kimchi*

The Chinese cabbage was cut into 4 pieces and soaked in 10% brine at 5°C for 12 hrs and then rinsed three times with tap water. The standardized ratios of the ingredients for *kimchi* were 13.0 of radish, 2.0 of green onion, 3.5 of red pepper powder, 1.4 of garlic, 0.6 of ginger, 2.2 of anchovy juice, and 1.0 of sugar in proportion to 100 of salted Chinese cabbage and the final salt concentration was adjusted to 2.5% (15). GK1, GK2, GK3 and GK4 of green tea leaves added *kimchi* (GK) containing 1, 2, 3 and 4 of dried green tea leaves in proportion to 100 of salted Chinese cabbage was made by the above *kimchi* (control *kimchi*, CK) recipe.

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Chinese cabbage (Kimhae, Korea), garlic, radish, green onion, ginger, red pepper powder (Yungyang NongHyup), anchovy juice (Daesang Co., Seoul) and salt (Guen salt from Sannaedle Co., Seoul) were purchased from a local market in Pusan, Korea. Dried green tea leaves (GTL) were obtained from Dahyangsanbang Co. (Sanchung, Kyungsang province).

The prepared *kimchi* (0 day) was packed in an alluminium pouch and then fermented for 14 days at 10°C. Optimally ripened *kimchis* (pH 4.3) were blended with stomacher (Seward, Stomacher 400, USA). The filtrates were centrifuged at 10,000 rpm for 20 minutes and supernatants were sterilized by passing then through 0.45 μm membrane filter (Millipore Products division, Bedford, MA) and the filtered juices were kept at -20°C for further experiments.

Determination of pH and acidity

The *kimchi* samples were blended with stomacher and the pH of the filtrate was determined using a pH meter (Corning Lab. Science Co., pH meter 220, USA). The acidity was determined according to the method of A.O.A.C. (16); 0.1% phenolphthalein was dropped into the *kimchi* filtrate and titrated with 0.1 N NaOH, and then the lactic acid content was calculated and expressed as the total acidity (%).

Enumeration of lactic acid bacteria

The filtrates obtained above were diluted with 0.1% (w/v) peptone solution and inoculated on modified *Lactobacillus* selection (m-LBS) agar medium containing 3.5% (w/v) sodium acetate and 0.25% (v/v) acetic acid for *Lactobacillus* sp. and phenylethyl alcohol sucrose (PES) agar medium containing 0.25% (v/v) phenylethyl alcohol and 2% sucrose for *Leuconostoc* sp. Colonies were counted (CFU/mL) after incubation for 3 days at 30°C and 5 days at 20°C for m-LBS and PES, repectively (17).

Antimutagenic test

Ames test

Aflatoxin B_1 (AFB_1) purchased from Sigma chemical Co. (St. Louis, Mo, USA) was dissloved in dimethyl sulfoxide (DMSO). *Salmonella typhimurium* TA100 bacterial strain, histidine requiring mutant, was provided by Dr. B.N. Ames (Univ. of California, Berkeley, USA) and was maintained as described by Maron and Ames (18). The genotype of the test strain was checked routinely for the histidine requirement, deep rough (*rfa*) character, UV sensitivity (*uvr* B mutation) and the presence of R factor. S9 mixure to activate the indirect mutagen, AFB_1, was also prepared by the method of Maron and Ames (18). Antimutagenicity test (19) was carried out according to a modified plate incorporation test (liquid preincubation of the organism with the test compound). In the preincubation test, 0.5 mL of S9 mixture, 0.1 mL of cell suspension of *Salmonella typhimurium* TA100 and 50 μL of AFB_1 in the absence or presence of *kimchi* juice (100 μL or 200 μL) were distributed into sterilized capped tube on an ice bath (18). The prepared sample mixture was preincubated at 37°C for 20 min, and then 2 mL of molten top agar supplemented with L-histidine and D-biotin at 45°C was added to the mixtures, gently mixed, and then poured onto minimal glucose agar plates (20). The plates were inverted and incubated at 37°C for 48 hr and then the revertant bacterial colonies were counted.

SOS chromotest

The modified assay method described by Quillardet and Hofnung (21), and Baik and Ham (22) was employed for SOS chromotest. Fifty μL of frozen stock of *E. coli* PQ37 was added to 5 mL/L medium and incubated in shaking water bath at 37°C overnight, then it was added to the 5 mL/L medium at 37°C and incubated in shaking water bath for 2 hrs until the absorbance at 660 nm reached 0.3~0.4, the active culture. The obtained active culture was diluted to 10 fold with L medium. One hundred μL of the diluted culture was distributed to the 2 series in the wells of a 96-well plate. Twenty μL of juices from *kimchi* that was treated with mutagen (10 μL juices from *kimchi* + 10 μL MNNG) were added, and then the SOS response was induced at 37°C for 90 min. One hundred μL of ONPG (o-nitrophenyl-β-D-galactopyranoside) and 100 μL of PNPP (p-nitrophenyl phosphate disodium) were added to each set of the wells to determine the activities of β-galactosidase (β-G) and akaline phosphatase (A-P), respectively.

After the color was allowed to develop for 30 min, 100 μL of 1.5 M Na_2CO_3 and 50 μL of 1 M HCl were added to stop the color developments of β-G and A-P, respectively After 5min, 50 μL of 2 M Tris buffer was added to the A-P to neutralize the HCl and then the SOS responses were determined at 420 nm. The SOS responses of the samples were calculated by the method of Miller (23).

Statistical analysis

All experiments were carried out in triplicate and means± standard deviation are reported. The data were analyzed by analysis of variances. Significant differnces between treatment means were determined by using Duncan's multiple range test.

RESULTS AND DISCUSSION

The *Kimchi* fermentation pattern is affected by several factors such as salt concentration, temperature, pH, related microorganisms, etc (24). In order to study the fermentation pattern of *kimchi* with the addition of GTL, changes in pH, acidity and the levels of lactic acid bacteria such as *Leuconostoc* sp. and *Lactobacillus* sp. of control *kimchi* (CK) and the GTL added *kimchis* (GKs) were investigated. Changes in pH and acidity of CK and GKs during fermentation at 10°C are shown in Fig. 1. Though the fermentation pattern in CK and GKs was similar, the fermentation period in GKs was longer than CK. The fermentation period of the GKs was prolonged as the added amount of GTL increased. The initial pHs of the CK and GKs were similar (CK for 5.59 and GKs for 5.53~5.68), however, the time to reach the optimally ripened status of *kimchi* (pH 4.3) was different among the *kimchi* samples. CK took 6 days, while GK1, GK2, GK3 and GK4 took

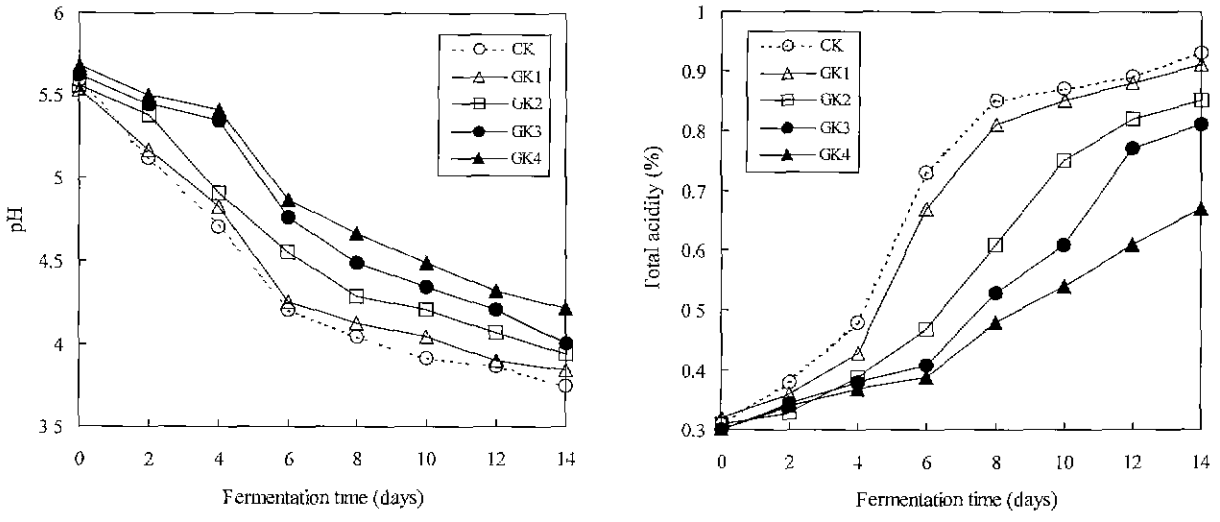

Fig. 1. Changes in pH and acidity of control *kimchi* (CK[1)]) and green tea leaves added *kimchis* (GKs[1)]) during fermentation at 10°C. [1)]See Materials and Methods.

6, 10, 12 and 14 days to reach the pH of 4.3, respectively. The trend of changes in the total acidity was the opposite to the pattern of those of pH. The initial acidity expressed as total acidity in CK and GKs was similar at 0.30~0.32%, but the time to reach optimally ripened status of *kimchi* (acidity 0.7~0.8%) was different. Park et al. (25) reported that the addition of dried green tea leaves to *kimchi* extended optimal edible periods more than 7 days when *kimchi* was fermented at 14°C.

Lactic acid bacteria (LAB) are the main microorganisms responsible for ripening of *kimchi*. *Leuconostoc mesentroides* initiates the fermentation of *kimchi* and is the predominant LAB in the early fermentation stages (26,27). As the pH drops to 4.6~4.9, *Leuconostoc mesentroides* is relatively inhibited, but other LAB such as *Lactobacillus brevis, Pediococcus cerevisias,* and *Lactobacillus plantarum* continue the fermentation process (26-28). The patterns of microbial changes in *Leuconostoc* sp. and *Lactobacillus* sp. in this study were similar between CK and GKs, but the time to reach the maximun level of the population was different (Fig. 2). The growth of *Leuconostoc* sp. and *Lactobacillus* sp. in GKs was delayed compared to those in CK. Among GKs, as the addition level of GTL increased, the growth of LAB was retarded. It was reported that the growth of *Lactobacillus brevis* and *Pediococcus pentosaceus* from *kimchi* was retarded by the addition of water extracts from green tea (29). Yeo et al. (30) also reported the tea extracts from green tea, oolong tea and black tea had the antimicrobial effects. Among the tea extracts, extracts of steamed green tea and roasted green tea and oolong tea showed higher antimicrobial activity than that of black tea. Because of the antimicrobial activity of green tea, GTL added *kimchi* seems to be delayed the fermentation period.

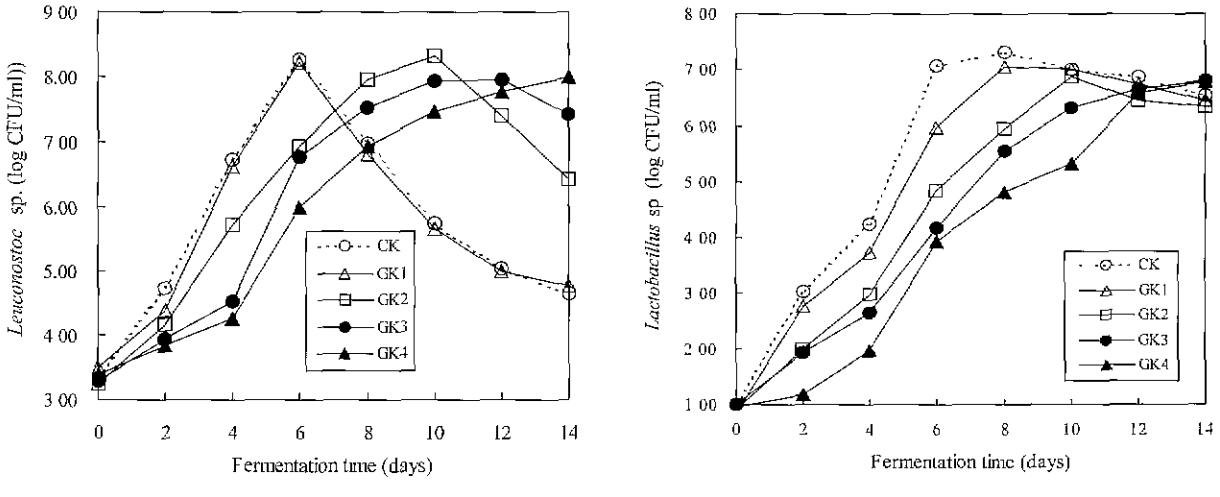

Fig. 2. Changes in the number of Lacic acid bacteria, *Leuconostoc* sp. and *Lactobacillus* sp. of control *kimchi* (CK[1)]) and green tea leaves added *kimchis* (GKs[1)]) during fermentation at 10°C. [1)]See Materials and methods

The juices from the CK and GKs showed antimutagenic activity against AFB_1 in *Salmonella typhimurium* TA100. In this study, the inhibition rate of CK was 37% at 100 μL/plate, while inhibition rates of GK1, GK2, GK3 and GK4 were 41%, 50%, 54%, and 57%, respectively ($p<0.05$). At the addition level of 200 μL/plate, the inhibition rate of CK was 49%, but the rates were 52%, 65%, 71% and 76% for GK1, GK2, GK3 and GK4 respectively (Table 1). It means that GKs have a higher antimutagenic activity than CK. Park et al. (2) reported that *kimchi* showed the antimutagenic activity against AFB_1 in the Ames test and MNNG in the SOS chromotest. Chung et al. (31) reported that green tea showed antimutagenic effects against both direct and indirect mutagen (MNNG and AFB_1) induced mutagenicity in the Ames test. As shown in Table 2, the *kimchi* samples showed an inhibitory effect on SOS response in *E. coli* PQ37. Especially, 44%, 52%, 60% and 70% of SOS response induced by MNNG were blocked by adding 10 μL/assay of juices from GK1, GK2, GK3 and GK4, respectively but CK showed 36% inhibition rate. From these studies, it can be concluded that GTL addition to the *kimchi* increases the antimutagenic acticity of the *kimchi*. Several studies have shown that *kimchi* had antimutagenic and anticancer effects (1,2) because of its various kinds of components in *kimchi* such as lactic acid bacteria, vitamin C, carotenoids, dietary fiber, etc. One of the active antimutagenic compounds we found in *kimchi* was β-sitosterol. A *kimchi* fraction that containing β-sitosterol showed *in vitro* antimutagenic activity in the Ames test using *Salmonella typhymirium* TA100 (32). There was also a report that *kimchi* has *in vivo* antimutagenicity in the *Drosophila* wing hair spot assay system (1). The green tea samples have been shown to prevent the formation of benzo[a]pyrene induced forestomach and lung tumorigenesis in mice (33). Also a study has shown that green tea consumption can inhibit the growth of established skin papillomas in mice (34). Therefore, each sample of *kimchi* or green tea showed strong antimutagenic and anticancer activities by itself. There is a report that catechins from green tea contribute to the characteristic bitter and an astringent taste of tea along with the brothy and sweet taste from amino acids such as glutamic acid and arginine (35). Because of the bitterness from tea polyphenols, GTL added *kimchi* had astringent and bitter taste as the amount of green tea leaves in *kimchi* increased. Though GTL added *kimchi* showed strong preservative effects and the antimutagenicity, the typical bitterness from green tea still remained when the green tea leaves were added into *kimchi*. In order to produce *kimchi* for commecial use, studies about decreasing the bitter taste, and animal tests demonstrating anticancer effects are still needed.

Table 1. Antimutagenic effect of juices from control *kimchi* (CK) and green tea leaves (GTL) added *kimchis* (GKs) on the mutagenicity induced by aflatoxin B_1 (AFB_1, 0.45 μg/plate) in *Salmonella typhimurium* TA100

Sample		Revertants/plate	Inhibition rate (%)
Spontaneous		110±6.7	
Control (AFB_1)		1002±19.3[a]	
AFB_1 +			
100 μL/plate	CK[1)]	675.7±17.9[b]	37
	CK+1%GTL (GK1)[1)]	638.3±16.7[c]	41
	CK+2%GTL (GK2)	559.7±16.5[d]	50
	CK+3%GTL (GK3)	522.7±16.5[ef]	54
	CK+4%GTL (GK4)	496.3± 6.4[f]	57
200 μL/plate	CK	570.0±30.0[d]	49
	CK+1%GTL (GK1)	537.3±30.1[de]	52
	CK+2%GTL (GK2)	429.0±32.5[g]	65
	CK+3%GTL (GK3)	375.0±13.2[h]	71
	CK+4%GTL (GK4)	323.7±25.1[i]	76

[a-i]Means with the different letters are significantly different at the $p<0.05$ level by Duncan's multiple range test

[1)]The ratio of ingredient and preparation method are shown in material and method. CK and GKs were fermented at 10°C until to reach the pH 4.3

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Table 2. SOS response of juices (10 μL/assay) from control *kimchi* (CK) and green tea leaves (GTL) added *kimchis* (GKs) fermented at 10°C against N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, 70 ng/assay) in *E. coli* PQ37

Sample	β-galactosidase (β)		Alkaline phosphatase (ρ)		(β)/(ρ)[1)]	SOS induction factor	Inhibition rate (%)
	OD_{420}	Unit[2)]	OD_{420}	Unit			
Spontaneous	0.623±0.12	20.7	0.552±0.02	18.4	1.12	1.00	
Control (MNNG)	1.154±0.03	38.5	0.532±0.01	17.7	2.17	1.94	
MNNG+							
CK[3)]	0.998±0.05	33.3	0.557±0.03	18.6	1.79	1.60	36
GK1[3)]	0.967±0.08	32.2	0.564±0.03	18.8	1.71	1.53	44
GK2	0.888±0.06	29.6	0.547±0.02	18.2	1.62	1.45	52
GK3	0.821±0.02	27.4	0.534±0.02	17.8	1.54	1.37	60
GK4	0.789±0.02	26.3	0.539±0.02	18.0	1.46	1.31	67

[1)]β-galactosidase/alkaline phosphatase

[2)]Enzyme unit = $\frac{1000 \times A_{420}}{\text{time}}$

[3)]The ratio of ingredient and preparation method are shown in material and method. CK and GKs were fermented at 10°C until to reach the pH 4.3

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