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Full Length Research Paper

Selection of probiotic strains and development of green tea based probiotics for livestock

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Twenty six (26) strains of *Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus casei, Enterococcus faecium, Bacillus subtilis, Bacillus coagulans,* and *Saccharomyces cerevisiae* from Korean Collection for Type Cultures (KCTC) were evaluated in this experiment for the development of green tea based probiotics production and their inhibition effect against pathogenic bacteria was examined. The strains were first tested for acid and bile tolerance, and the higher tolerance strains were subsequently analyzed for heat tolerance and susceptibility to green tea extracts. Among the tested strains, *L. acidophilus* KCTC 3111, *L. plantarum* KCTC 3104, *B. subtilis* KCTC 3239, *B. coagulans* KCTC 1015, and *S. cerevisiae* KCTC 7915 which displayed a high tolerance to acid, bile, and heat, and no susceptibility to green tea extract, were selected to produce fermented green tea probiotics. Associative effects of green tea probiotics and *Escherichia coli* S93 F5, S99 LT, S170 F41, and S171 F6 revealed that *E. coli* numbers had gradually decreased compared to the control. Stability results of *Lactobacillus* spp. and *Bacillus* spp. in green tea probiotics showed that they were stable at 10^8 CFU/g during eight weeks of storage. Therefore, it is expected that green tea probiotics may provide a potential feed additive for livestock.

Key words: Green tea, probiotics, tolerance, *E. coli*, feed additive, livestock.

INTRODUCTION

Probiotics have been defined as mono or mixed cultures of live microorganisms which when administered into diet of animal or man in adequate amounts, make a health benefit to the host (FAO/WHO, 2001). Due to the appearance of antibiotic-resistant bacteria and antibiotic residue in livestock products, the use of probiotics instead of antibiotics has been strongly recommended (Snyder and Champness, 1997). Probiotics have been paid major attention from researchers in recent years. Several studies have been performed to determine the effects of probiotic microorganisms using different formulas for the purposes of preventing or treating diseases (Mercenier et al., 2002). In animal nutrition, probiotics are used as feed additives as one of the three different groups; lactic acid bacteria (Taheri et al., 2009), *Bacillus* spores (Anadon et al., 2006), or yeasts (Shareef and Al-Dabbagh, 2009). These groups differ from one another in their properties, origin, and mode of action. The main activities of probiotics are maintenance and reconstitution of intestinal microflora equilibrium which is achieved by various modes of action. The gut microflora have been shown to be involved in protection against a variety of pathogens including *Escherichia coli*, *Salmonella*, *Campylobacter*, *Clostridium*, and rotavirus. Hence, probiotics may provide an effective preventative and therapeutic treatment for these infections (Fuller, 1999).

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The expected beneficial characteristics of probiotic strains fall into five basic categories: physiological, immunological, metabolic and genetic traits, and technological properties. Potential probiotic strains must include characteristics such as nontoxicity, nonpathogenicity, ability to modulate immune responses, and production of antimicrobial substances (Lim and Im, 2009). In addition, they should be able to survive and proliferate at the target site, resist gastric acid and bile, adhere to the gastrointestinal tract, and antagonize pathogenic bacteria (Mercenier et al., 2002). The use of probiotic bacteria and their metabolites has shown many beneficial effects in cattle, pigs, and chickens. These advantages include the improvement of general health, feed conversion ratios, and growth rate, as well as resistance to diseases (Fuller, 1992).

Another developed non-antibiotic method in the use involves functional medicinal plants. Green tea (*Camellia sinensis*) has been used for centuries by Korean, Japanese, and Chinese people as an anti-aging herb and its polyphenolic compounds which are mainly composed of seven types of catechins are reported to have, anticarcinogenic (Mukhtar and Ahmad, 1999), antimicrobial (Hara–Kudo et al., 2005), and antioxidant (Nishida et al., 2006) in animals. In addition to human consumption, low-grade green tea has been used as an ingredient in feed for livestock (Cao et al., 2005; Suzuki et al., 2002) with positive effects on animal performance. Recently, there has been a growing interest among researchers and the feed industry to develop a probiotic feed containing a combination of beneficial microbial strains and medicinal plants. As a result, this study was conducted to select appropriate probiotic strains to use with green tea. Tests, such as acid, bile, and heat tolerance, and susceptibility to green tea extract as well as inhibitory effects on pathogenic bacteria, and stability under storage conditions were also conducted.

MATERIALS AND METHODS

Microbial strains

A total of 26 strains, including *Lactobacillus acidophilus*, *L. plantarum*, *L. casei, Enterococcus faecium, Bacillus subtilis*, *B. coagulans,* and *Saccharomyces cerevisiae* were used to select appropriate probiotic strains. These microbes are part of the Korean Collection for Type Cultures (KCTC) and were obtained from the Korea Research Institute of Bioscience and Biotechnology. The culture media for the experimental strains were de Man, Rogosa, and Sharpe (MRS) medium (Difco, Detroit, MI, USA) for *Lactobacillus* spp., nutrient broth (NB) medium (Difco) for *Bacillus* spp., and yeast and mold (YM) medium (Difco) for yeast (Table 1).

Tolerance measurement

To test the acid tolerance of the strains, simulated gastric juice was made according to a slightly altered method of Kobayashi et al. (1974). Experimental strains were injected into sterilized broth media (50 ml) and then cultivated at 37°C for 48 h. Each strain (1 ml) was inoculated into simulated gastric juice consisting of NB medium containing 1000 U pepsin/ml adjusted to pH 3 using 1N HCl (50 ml), and also into sterilized NB medium, pH 7 (50 ml) as a control. *Lactobacillus* spp. and *Bacillus* spp. were incubated at 37°C and yeast at 30°C for 3 h. Serial decimal dilutions in MRS agar medium for *Lactobacillus* spp., nutrient agar (NA) medium for *Bacillus* spp., and YM agar medium for yeast were prepared and aliquots (1 ml) from the dilutions were then spotted on MRS, NA, or YM agar plates to determine the number of surviving cells. The experiment was repeated three times. Bile tolerance was measured according to a slightly altered method of Park (1999). For analysis of bile tolerance, 1 ml of inoculation liquid of each strain was injected into sterilized NB medium with 0.3% hog bile acid or into NB medium without hog bile acid as a control. To count bile acid tolerant cells, the same methods were used as for measuring acid tolerance. Only those experimental strains that were confirmed to have strong acid and bile tolerance in the initial tests were selected for heat tolerance testing. Heat tolerance was tested according to a slightly altered method of You et al. (2005). *Lactobacillus* spp. and *Bacillus* spp. were treated with heat at 80°C for 15 min, and 70°C for 5 min in the case of yeast. Heat tolerant cells were counted using the same method used for acid tolerance described previously.

Susceptibility of selected strains to green tea extract

According to the method of Yun et al. (1996), green tea was extracted in a water bath at 80°C for 30 min by adding 100 ml of distilled water to 100 mg of green tea powder, after which 10, 20, 30, 40, and 50% green tea extract solutions were prepared in Tryptic Soy Agar (TSA) media (Difco). Selected strains that had strong acid, bile, and heat tolerance were inoculated (1 ml) into TSA media containing each of the prepared green tea extract solutions and cultured at 37°C for 48 h. The cultures were compared to those grown in TSA media without green tea extract (control) and each strain was defined as 'resistant (+)' if it grew well, 'intermediate (w)' if it did not grow well, or 'susceptible (-)' if it did not grow at all.

Production of green tea probiotics

Strains selected for tolerance to acid, bile, heat, and green tea extract were used for preparation of green tea probiotics. The process of producing probiotics in this experiment was the same method as applied in industry. We used a commercial fermentor (Model: W-1000, Wonbalhyo Industry Co., Korea) for anaerobic and aerobic fermentation process. Defatted rice bran and wheat bran, which are typically used in the industrial field, were used for fermented solid media (Lee, 2006). Green tea probiotics were produced as follows: *L. acidophilus* KCTC 3111 and *L. plantarum* KCTC 3104 were formulated into media containing 30% green tea, 50% defatted rice bran, and 20% wheat bran. The culture was fermented at 40°C repeating 5 h of static and 3 h of shaking conditions for two days. There was a second inoculation with *B. subtilis* KCTC 3239, *B. coagulans* KCTC 1015, and *S. cerevisiae* KCTC 7915, and continued the process for three days. The number of cells in the green tea probiotics was analyzed as well. The green tea probiotics (1 g) were diluted with sterilized distilled water (10 ml) at room temperature for approximately 10 min, then 1 ml was diluted 10-fold serially in 0.85% NaCl solution, cultured, and the number of colonies counted. Each strain inoculation was made with 10^{10} CFU/g.

Table 1. Media and culture methods used for selection of microbial strains.

Microbial strain[1]	Medium	Culture method	Culture time (h)
Lactobacillus acidophilus KCTC 3111	MRS	Anaerobic	48
L. acidophilus KCTC 3146			
L. acidophilus KCTC 3149			
L. acidophilus KCTC 3150			
Lactobacillus casei KCTC 2180	MRS	Anaerobic	48
L. casei KCTC 3109			
Lactobacillus plantarum KCTC 1048	MRS	Anaerobic	48
L. plantarum KCTC 3099			
L. plantarum KCTC 3104			
L. plantarum KCTC 3107			
Enterococcus faecium KCTC 2022	MRS	Anaerobic	48
E. faecium KCTC 3078			
E. faecium KCTC 3080			
E. faecium KCTC 3122			
Bacillus subtilis KCTC 1022	NB	Aerobic	48
B. subtilis KCTC 1103			
B. subtilis KCTC 1666			
B. subtilis KCTC 3239			
Bacillus coagulans KCTC 1013	NB	Aerobic	48
B. coagulans KCTC 1014			
B. coagulans KCTC 1015			
B. coagulans KCTC 1662			
Saccharomyces cerevisiae KCTC 1201	YM	Anaerobic	24
S. cerevisiae KCTC 7107			
S. cerevisiae KCTC 7915			
S. cerevisiae KCTC 7928			

[1]Korean Collection for Type Cultures (KCTC) strains obtained from the Korea Research Institute of Bioscience and Biotechnology (KRIBB). MRS = de Man, Rogosa and Sharpe; NB = nutrient broth; YM = yeast and mold (Difco, USA).

Chemical composition analysis

The green tea used in this experiment was provided by the Tea Experimental Station (Bosung, Korea). All proximate components of green tea and green tea probiotic were determined by the methods of the Association of Official Analytical Chemists (AOAC, 1990). The method devised by Ikeda et al. (2003) was used for catechin analysis. Briefly, approximately 100 mg of green tea and green tea probiotic samples were dissolved in 100 ml of distilled water and heated in a water bath at 80°C for 30 min. After cooling, the solution was filtered and 25 ml was transferred to a separating funnel and mixed three times with 25 ml of chloroform. The water layer was collected and extracted with 25 ml of ethyl acetate. The ethyl acetate extract was then transferred to a rotary evaporator and evaporated at 30°C and 5 ml of methanol was added after which it was filtered through a membrane filter (0.45 µm PVDF) and Sep-Pack C18 cartridge. The content of catechin compounds from the extracts was determined using a high pressure liquid chromatography (HPLC, Model 501; Waters, Milford, MA, USA).

Growth inhibition of pathogens

Growth inhibition of pathogens was measured according to a slightly altered method of Park (1999) and Kim et al. (2002). The experiment used four strains of *E. coli;* S93 F5, S99 LT, S170 F41, and S171 F6 obtained from National Veterinary Research and Quarantine, Korea. Each pathogenic strain was cultured anaerobically at 37°C for 24 h in 50 ml TSB media (Difco), then 1 ml of culture, 1 g of green tea probiotic powder, and the selected strains were inoculated together into a new 50 ml volume of sterilized NB media and associatively incubated in anaerobic conditions at 37°C for 24 h. *E. coli* density was measured every 3 h and results were compared to a control group of *E. coli* that was cultured alone under the same conditions. MacConkey Sorbitol Agar (Difco, Detroit, MI, USA) was used as *E. coli* selective media.

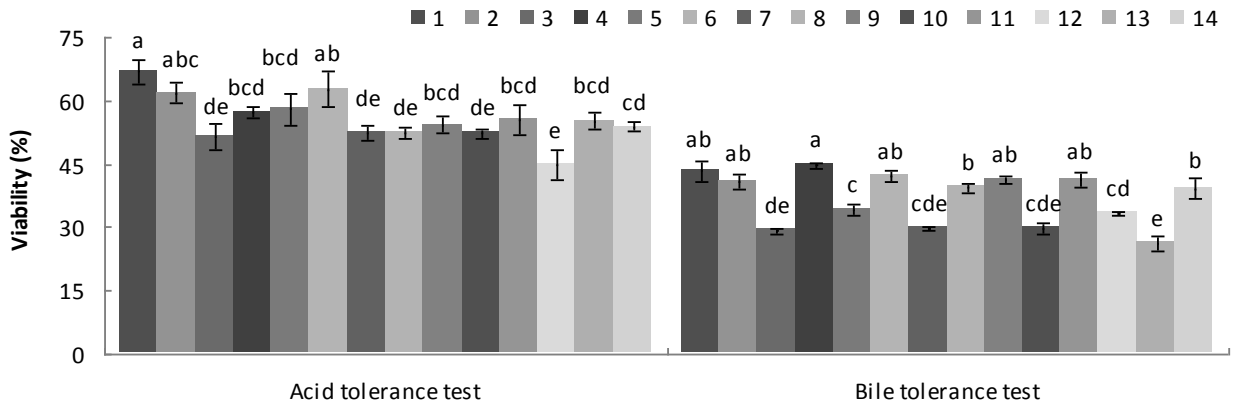

Figure 1. Effect of simulated acid and bile on the growth of lactic acid bacteria**.** The microbial strains: 1, *L. acidophilus* KCTC 3111; 2, *L. acidophilus* KCTC 3146; 3, *L. acidophilus* KCTC 3149; 4, *L. acidophilus* KCTC 3150; 5, *L. casei* KCTC 2180; 6, *L. casei KCTC* 3109; 7, L. plantarum KCTC 1048; 8, *L. plantarum* KCTC 3099; 9, *L. plantarum* KCTC 3104; 10, *L. plantarum* KCTC 3107; 1, *E. faecium* KCTC 2022; 12, *E. faecium* KCTC 3078; 13, *E. faecium* KCTC 3080; 14, *E. faecium* KCTC 3122. Values are expressed as means ± SE. Bars within the acid or bile tolerance test having uncommon letters indicate significant differences (a > b > c > d, P<0.05).

Stability of probiotic strains under storage conditions

Manufactured green tea probiotics (1 kg) were put into vacuum packing and stored for eight weeks at room temperature to estimate microorganism number of green tea probiotics during storage. Every week 1 g was removed and the number of microorganisms was counted. The same *Lactobacillus* and *Bacillus* strains, and defatted rice bran were fermented and compared with green tea probiotics. Serial decimal dilutions in MRS agar medium (Difco) for *Lactobacillus* spp. and NA medium (Difco) for *Bacillus* spp. were prepared and aliquots (1 ml) of the dilutions were spotted to determine colony numbers on MRS and NA agar plates, respectively.

Statistical analysis

All the tests were repeated three times and the data were analyzed using the GLM procedure of SAS (2003). Microbial numbers were logarithmically transformed (log_{10} CFU/mL or log_{10} CFU/g) before analysis. Viability was expressed as percentage and calculated from original microbial numbers, and means were separated by Duncan's multiple range test. All data are presented as mean values ± SE. The threshold for statistical significance was P<0.05.

RESULTS AND DISCUSSION

Acid, bile, and heat tolerance of microbial strains

The effects of simulated gastric juice and bile acids on the growth of lactic acid bacteria are shown in Figure 1. The viability of lactic acid bacteria in simulated gastric juice and bile acid varied among species and strains. *L. acidophilus* KCTC 3111, *L. acidophilus* KCTC 3146, *L. acidophilus* KCTC 3150, *L. casei* KCTC 3109, *L. plantarum* KCTC 3104, and *E. faecium* KCTC 2022 exhibited the highest tolerance to acid (>50%) and bile (>40%). Acid and bile tolerance of *Bacillus* spp. and *S. cerevisiae* strains were studied and high survival rates (>40%) of *B. coagulans* KCTC 1015, *B. subtilis* KCTC 3239, *S. cerevisiae* KCTC 7915, and *S. cerevisiae* KCTC 7928 were observed following exposure to simulated gastric juice and bile acid (Figure 2). A total of 10 strains with relatively strong resistance to acid and bile were selected and tested for heat tolerance (Figure 3). All strains showed their heat tolerance to be between 35.86% and 54.74%. *S. cerevisiae* KCTC 7915 exhibited the highest heat tolerance while *L. acidophilus* KCTC 3146 was lowest.

The microflora of the gastrointestinal tract plays a crucial role in the anatomical, physiological, and immunological development of the host by stimulating the immune system to respond rapidly to infection by pathogens and inhibiting colonization of the gut by harmful or pathogenic bacteria through bacterial antagonism (Cebra et al., 1999). Acid and bile tolerance are two fundamental properties that indicate the ability of probiotic microorganisms to survive passage through the gastrointestinal tract, resisting the acidic conditions in the stomach and the bile acids at the beginning of the small intestine (Strompfová and Lauková, 2007). The results of these tests are predictive of the ability of the strains to survive in acidic products. Garriga et al. (1998) screened lactic acid bacteria with regard to pH 3 tolerance, and showed that pH 3 did not decrease the number of lactic acid bacteria. Tolerance to bile salts was initially associated with the presence of bile salt hydrolase activity (Taranto et al., 2006). The *L. plantarum* NCIMB 8826 strain was shown to be acid and bile tolerant

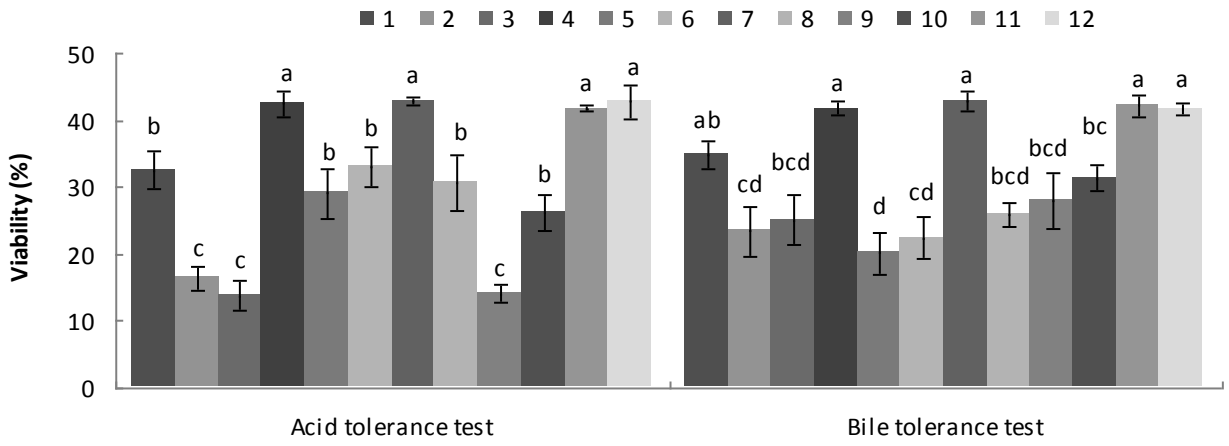

Figure 2. Effect of simulated acid and bile on the growth of *Bacillus* spp. and *Saccharomyces* spp. The microbial strains: 1, *B. subtilis* KCTC 1022; 2, *B. subtilis* KCTC 1103; 3, *B. subtilis* KCTC 1666; 4, *B. subtilis* KCTC 3239; 5, *B. coagulans* KCTC 1013; 6, *B. coagulans* KCTC 1014; 7, *B. coagulans* KCTC 1015; 8, *B. coagulans* KCTC 1662; 9, *S. cerevisiae* KCTC 1201; 10, *S. cerevisiae* KCTC 7107; 11, *S. cerevisiae* KCTC 7915; 12, *S. cerevisiae* KCTC 7928. Values are expressed as means ± SE. Bars within the acid or bile tolerance test having uncommon letters indicate significant differences (a > b > c > d, P<0.05).

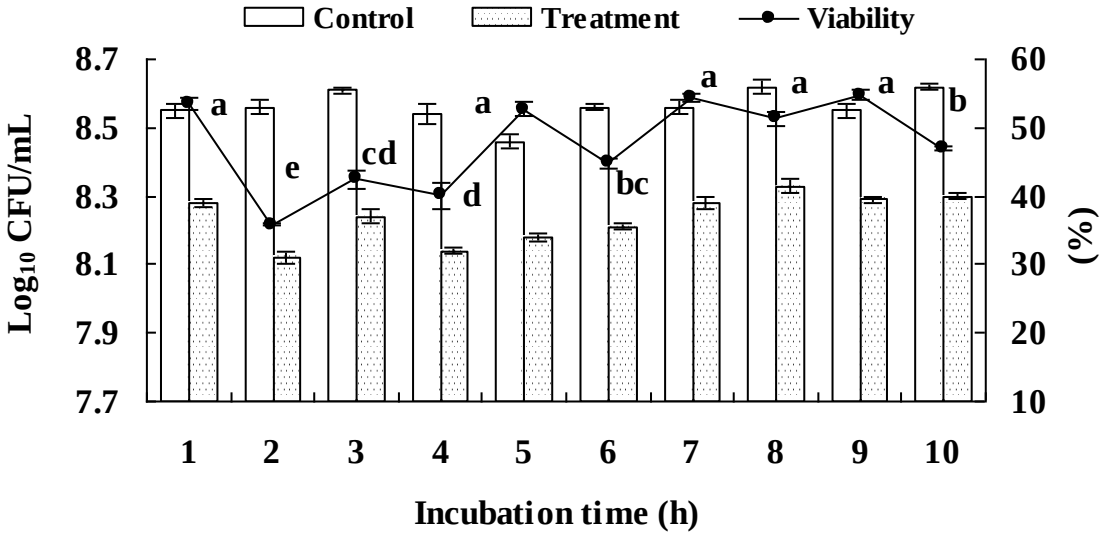

Figure 3. Effect of heat treatment on selected microbial strains. Microbial strain: 1, *L. acidophilus* KCTC 3111; 2, *L. acidophilus* KCTC 3146; 3, *L. acidophilus* KCTC 3150; 4, *L. casei* KCTC 3109; 5, *L. plantarum* KCTC 3104; 6, *E. faecium* KCTC 2022; 7, *B. subtilis* KCTC 3239; 8, *B. coagulans* KCTC 1015; 9, *S. cerevisiae* KCTC 7915; 10, *S. cerevisiae* KCTC 7928. Values are expressed as means ± SE. Within the viability of microbial strains, different letters indicate significant differences (a > b > c > d, P<0.05).

(Charalampopoulos and Pandiella, 2010), and has been shown to exert anti-inflammatory activities in animal models (Foligne et al., 2006). Survivability in acid and bile of the strain *L. acidophilus* (Liong and Shah, 2005), and lactic acid producing bacteria (Thirabunyanon et al., 2009) have been previously reported. Some species of *Bacillus* are already being used in food production, so their safety has been proven. Some strains of *B. coagulans* are able to survive against heat and acidity of the stomach and bile acids, although in general the strains of this species

Table 2. Susceptibility of bacterial strains after incubation with green tea extract.

Bacterial strain	Green tea extract (%)				
	10	20	30	40	50
L. acidophilus KCTC 3111	+	+	+	+	+
L. acidophilus KCTC 3146	+	+	+	w	–
L. acidophilus KCTC 3150	+	+	+	w	w
L. casei KCTC 3109	+	+	+	w	–
L. plantarum KCTC 3104	+	+	+	+	+
E. faecium KCTC 2022	+	+	+	w	w
B. subtilis KCTC 3239	+	+	+	+	+
B. coagulans KCTC 1015	+	+	+	+	+
S. cerevisiae KCTC 7915	+	+	+	+	+
S. cerevisiae KCTC 7928	+	+	+	w	w

+ = resistant; w = intermediate; – = susceptible.

are quite heterogeneous (De Vecchi and Drago, 2006). Strains with these qualities have an increased chance of survival through the gastrointestinal tract, thus allowing for transient population of the small and large intestines by *B. coagulans* (Adami and Cavazzoni, 1999). *S. cerevisiae* is considered as one of the live probiotic microorganisms that are administered through the digestive tract and had a positive impact on the host's health through its direct nutritional effect (Patterson and Burkholder, 2003). Heat treatment is important for the control purposes of pathogens (Leuschner and Bew, 2003). In addition, probiotic strains in feed are required to tolerate heat during the pellet making process (Conway and Kjelleberg, 1989). All selected strains showed average heat tolerance in this study.

Susceptibility of selected microbial strains to green tea extract and chemical composition of green tea probiotics

Incubation of selected bacterial strains with green tea extract are shown in Table 2. None of the selected strains showed susceptibility to green tea extracts of 10 to 30%, but there was susceptibility of some strains to 40 and 50% green tea extracts. From this experiment, *L. acidophilus* KCTC 3111, *L. plantarum* KCTC 3104, *B. subtilis* KCTC 3239, *B. coagulans* KCTC 1015, and *S. cerevisiae* KCTC 7915, which displayed no susceptibility to green tea extracts and had highest heat tolerance (>50%), were selected to make fermented green tea probiotics. Each strain inoculation was made with 10^{10} CFU/g and after fermentation and drying, the number of each strain was reduced 1-2 log cycles (Table 3). Chemical analysis results revealed that green tea probiotics had higher levels of moisture and crude ash but lower levels of crude protein, crude fat, crude fiber and catechin content compared to that of green tea alone (Table 3).

None of the selected strains showed susceptibility to green tea extracts of 10 to 30%; however, there was susceptibility to 40 and 50% extracts. Based on these findings, and consideration of the stability of strains selected to green tea components, we concluded that 30% would be the proper amount of green tea to use when producing green tea probiotics. From this experiment, *L. acidophilus* KCTC 3111, *L. plantarum* KCTC 3104, *B. subtilis* KCTC 3239, *B. coagulans* KCTC 1015, and *S. cerevisiae* KCTC 7915, which had no susceptibility to green tea extracts, were selected as the strains to make fermented green tea probiotics. It has been shown that some cereals, such as oats and maize, as well as extracts of barley, wheat, and malt are also suitable substrates for the growth of probiotic microorganisms and enhanced acid and bile tolerance (Charalampopoulos and Pandiella, 2010). Although the effects of cereal extracts and cereal fiber on gastrointestinal stability were different, but they displayed a synergistic effect on the gastrointestinal tolerance of *Lactobacillus* spp. (Michida et al. 2006). Generally, it has been suggested that in animals efficacy for most probiotics could be demonstrated with a daily intake of 10^8 to 10^9 microorganisms (Patterson and Burkholder, 2003). In the study, the number of bacteria in the green tea probiotics was maintained at 10^8 to 10^9 CFU/g. The total catechin content of green tea probiotics was 30% of green tea analyzed in this experiment. This result is likely derived from the fact that the mixture ratio of green tea was 30% in the green tea probiotics.

Growth inhibition of pathogens

The effects of green tea probiotics on growth inhibition of pathogens after associative inoculation are shown in Figure 4. Following associative inoculation of *E. coli* S93 F5, S99 LT, S170 F41 and S171 F6 with GTP, the total number of *E. coli* increased up to 6 h, but the numbers were distinctly lower compared to the individual *E. coli* groups. After 6 h of incubation, *E. coli* number of the GTP groups dramatically decreased up to the end of the experimental period (18 h) from 8.41 to 5.85 $\log_{10}$ CFU/mL for *E. coli* S93 F5, from 8.50 to 7.12 $\log_{10}$ CFU/mL for *E. coli* S99 LT, from 8.83 to 5.64 $\log_{10}$ CFU/mL for *E. coli* S170 F41 and from 8.75 to 6.15 $\log_{10}$ CFU/mL for *E. coli* S171 F6.

We observed a slow inhibitory effect on *E. coli* proliferation from the beginning when associatively cultured with green tea probiotics, but after 6 h of inoculation, inhibitory effect was markedly improved. The

Table 3. The number of microflora and chemical composition of green tea and green tea probiotics.

Microbial strain		Microbial number (CFU/g)
Lactobacillus acidophilus KCTC 3111		$3.2 \times 10^8 \pm 1.3 \times 10^7$
Lactobacillus plantarum KCTC 3104		$2.2 \times 10^8 \pm 1.2 \times 10^7$
Bacillus subtilis KCTC 3239 and *Bacillus coagulans* KCTC 1015		$4.5 \times 10^9 \pm 1.1 \times 10^8$
Saccharomyces cerevisiae KCTC 7915		$5.2 \times 10^8 \pm 2.4 \times 10^7$
Chemical composition (%, Dry matter basis)	**Green tea**	**Green tea probiotics**
Moisture	11.16 ± 0.56	15.00 ± 0.46
Crude protein	22.36 ± 0.55	17.20 ± 0.45
Crude fat	7.36 ± 0.40	4.93 ± 0.42
Crude fiber	16.20 ± 0.43	10.89 ± 0.55
Crude ash	6.22 ± 0.30	7.65 ± 0.36
Total catechin	16.16 ± 0.43	4.76 ± 0.33

Values are expressed as mean ± SE.

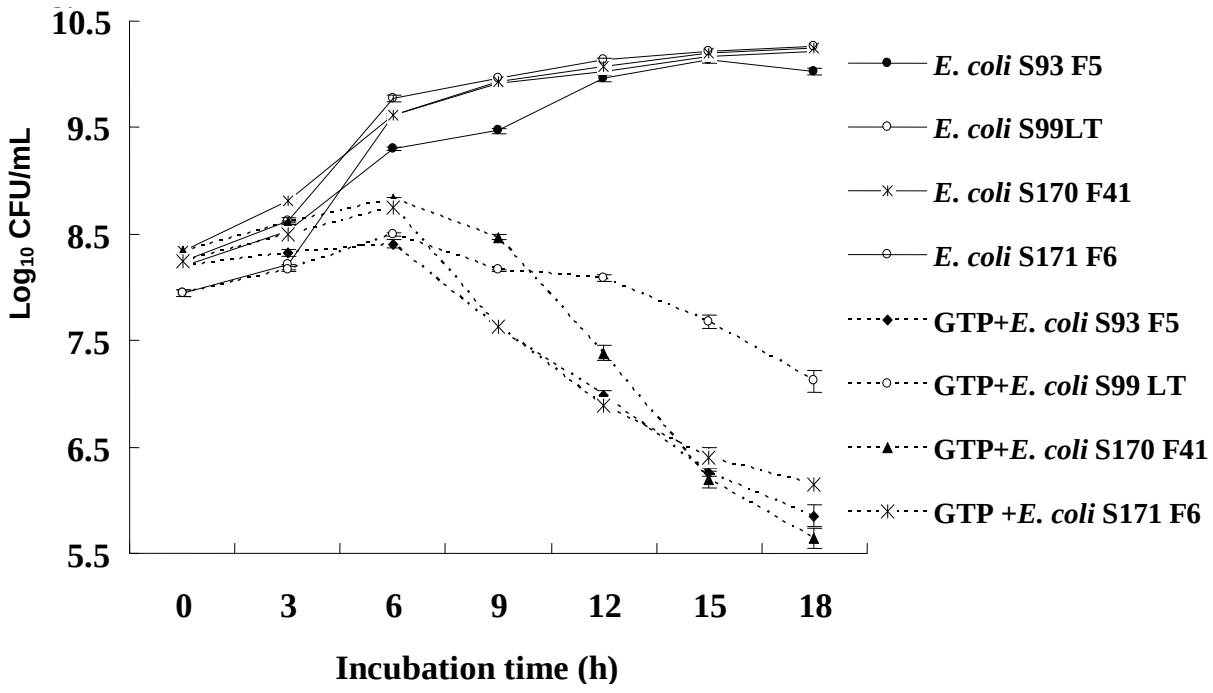

Figure 4. Growth of pathogenic *E. coli* strains without or with associative inoculation of green tea probiotics. Values are express as means ± SE. GTP = Green tea probiotics.

rationale behind this observation is that green tea probiotics inhibit pathogens due to the complementary effects of green tea and probiotic bacteria. These results are due to the proliferation of beneficial strains and the release of green tea compounds inside the culture fluid as time passes during the associative culture. It is well known that tea catechins are bactericidal and inhibit the growth of bacterial spores (Hara–kudo et al., 2005), but they do not affect lactic acid bacteria (Hara, 1997). Sugita-Konishi et al. (1999) reported that entero-

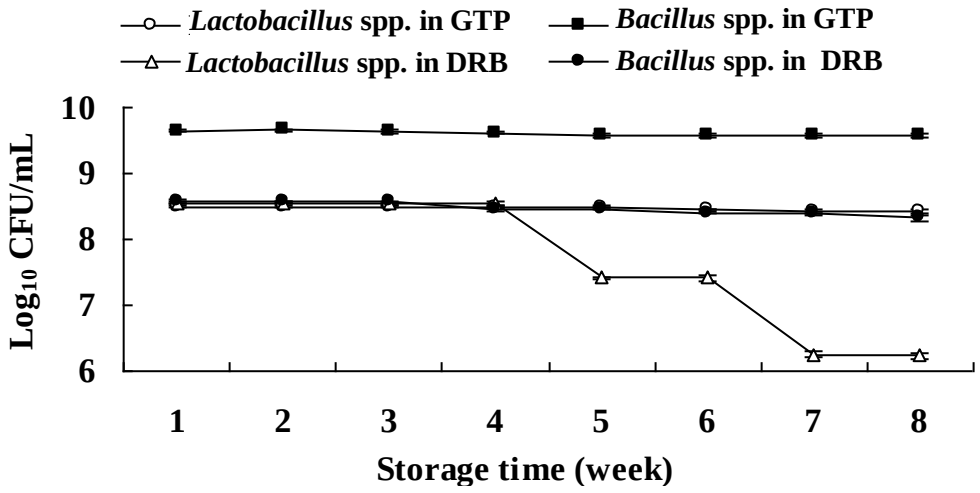

Figure 5. Stability of probiotic strains under storage conditions. Values are express as means ± SE. GTP = Green tea probiotics; DRB = Defatted rice bran.

hemorrhagic *E. coli* O157:H7 failed to produce verocytotoxin with low concentrations of green tea catechin. *L. acidophilus* decreases the pH of culture solutions and produces the antimicrobial compounds or antibiotics like acidolin, acidophilin, and lactacin-B (Havenaar et al., 1992). Green tea extracts also improved the microflora balance and showed antimicrobial effects against pathogenic bacteria in Holstein calves (Ishihara et al., 2001), and swine (Hara et al., 1995). According to the report of Kim et al. (2002), when *L. acidophilus* and *E. coli* were inoculated associatively, there was no change for 9 h, but 12 h after the inoculation, *E. coli* tended to decrease rapidly. Lee et al. (2002) showed that when lactic acid bacterial strains (K1, D2, F35-2) were co-cultured with *L. monocytogenes*, *S. typhimurium*, or *E. coli* O157:H7 in YS medium, the foodborne microorganisms were completely inhibited within 72 h of incubation at a ratio of 5:1 (isolate:pathogen). Timmerman et al. (2004) noted that multi-strain or multi-species probiotics were more effective than mono-strain probiotics concerning resistance to pathogens and performance in animals.

Stability of probiotic strains under storage conditions

Changes in the number of microbes in green tea probiotics based on storage period are shown in Figure 5. *Lactobacillus* spp. with green tea probiotics were stable at 10^8 $\log_{10}$ CFU/g for eight weeks starting from the first week. However, after culture with defatted rice bran, the *Lactobacillus* spp. were stable for only four weeks, then continuously decreased through eight weeks dropping by approximately 2 log cycles. *Bacillus* spp. included in defatted rice bran and green tea probiotics maintained a constant number of microbes from the first to the eight weeks of storage.

The survival of probiotics is influenced by various factors, but the storage period strongly affects the survival rate of microbes and the shelf life of products (Hamilton–Miller et al., 1996). The stability of microbes in green tea probiotics may be due to the effects of the green tea fermented medium. In the case of cereal-based fermented products containing probiotic strains, the most likely factors influencing probiotic survival during refrigerated storage are pH and lactic acid concentration of the fermented product. It has been shown that the levels of sugar and lactic acid in fermented products affect cell survival largely (Charalampopoulos and Pandiella, 2010).

Conclusion

The green tea probiotics tested in this study were multi-strain and exhibited a high tolerance to acid, bile, and heat. Therefore, it is essential to know that selected strains do not inhibit each other in the animal trial. To analyze these results more precisely, more research are needed. In this study, selected strains had no susceptibility to green tea extract and showed stability during eight weeks of storage. In addition, when antimicrobial effect was tested, green tea probiotics inhibited the proliferation of *E. coli* S93 F5, S99 LT, S170 F41, and S171 F6 strains. It is expected from the results that using green tea probiotics as a feed supplement may offer a substitute for the use of antibiotics in livestock nutrition.

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