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Lactobacillus plantarum G72 Showing Production of Folate and Short-chain Fatty Acids

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The aim of this study was to determine the production of folate, short chain fatty acids (SCFAs), and antimicrobial activity exhibited by *Lactobacillus plantarum* G72 for potential dietary application in pregnant women. *L. plantarum* G72 has been reported to possess characteristic activities and functionality including β-galactosidase activity and antioxidant activities. *L. plantarum* G72 showed antibacterial activity against pathogenic bacteria (*Listeria monocytogenes* ATCC 15313, *Salmonella typhimurium* P99, *Escherichia coli* ATCC 25922, and *Staphylococcus aureus* KCCM 11335) using a modified method, and formation of the largest inhibition zone was observed against *S. aureus* KCCM 11335 (12.0-17.0 mm). The adherence of four food-borne pathogenic bacteria to HT-29 cells was inhibited by *L. plantarum* G72 (0.13 to 0.92 log CFU/ml). The most considerable inhibition of adherence to HT-29 cells was observed by using *L. plantarum* G72 against *S.* typhimurim P99. Additionally, folate production by *L. plantarum* G72 was 50.1 ng/ml, and *L. plantarum* G72 produced relatively more lactic acid (11,176.73 mg/kg) than acetic, propionic, or butyric acids. Therefore, the results of this study suggest that *L. plantarum* G72 may serve as a multifunctional food additive in the health industry.

Keywords: **Probiotics, *Lactobacillus plantarum*, antimicrobial activity, folate, short-chain fatty acids**

Introduction

Probiotics are defined as “live micro-organisms”, when administered in adequate amounts to the host, confers a health benefit [1–3]. Probiotics should be able to tolerate the gastric condition of the gastrointestinal (GI) tract and reach the small intestine and adhere to human epithelial surfaces [4, 5]. Probiotics have various beneficial effects including antioxidant, anti-allergic, anticancer, cholesterol reduction, diarrhea prevention, and immune function enhancing activities [5–7]. Especially, probiotic microorganism is important to link to the gastrointestinal tract. The digestive viability and antimicrobial effects of probiotics are influenced by pH, acidity, temperature, removed hydrogen, and oxygen peroxide [9].

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Folate (vitamins B_9), is required for body function to repair and synthesize DNA [10], and is important for basic physiological mechanisms [11]. Therefore, folate is required not only for most people but also for pregnant women as a health functional food. Especially, pregnant women are advised to increase their folate intake as folate helps in the production of red blood cell and development of the brain and the spinal cord, thus, a sufficient of folate could promote fetal growth and development [12]. Recently, an interesting study about folate production by probiotic has been reported [13]. *Lactobacillus sakei* strains have indicated that the highest folate produced 730–1,484 ng/g [14]. In addition, probiotics could help absorb folate through the intestinal lining according to a research that reports intestinal microbiota to be a source of vitamins [15].

Short-chains fatty acids (SCFAs) contain fewer than six carbons, exist in straight and branched-chains [16]. Acetic acid, propionic acid, and butyric acid are reported

to be the most important SCFAs. SCFAs are produced by bacteria in the GI tract and these bacteria use non-digestible carbohydrates (NDC) as their main energy source [17, 18]. Especially, production of SCFAs is important to make the intestinal environment more acidic, thereby inhibiting the colony of pathogenic microorganisms [18]. Moreover, they have various functions, including anti-inflammatory, antifungal, and anti-obesity effects [20, 21].

In previous study, *Lactobacillus plantarum* G72 was isolated from kimchi and identified as *L. plantarum* by 16S rRNA sequencing [7]. *L. plantarum* G72 has been reported to have characteristic activities such as tolerance to artificial gastric conditions, enzyme production, antibiotic sensitivity, and adhesion to HT-29 cells. In addition, *L. plantarum* G72 of β-galactosidase activity and antioxidant activities that have been established through the DPPH free radical scavenging assay (21.08%) and the β-carotene bleaching assay (31.92%). Thus, the aim of this study was to investigate production of folate, SCFAs and antimicrobial activity by *L. plantarum* G72 for pregnant women.

Materials and Methods

Bacterial strains and culture conditions

L. plantarum G72 was isolated from kimchi and *Lactobacillus rhamnosus* GG (LGG) was obtained from the Korean collection for type cultures (Jeolla-do, Korea). They were grown in lactobacilli MRS broth (BD BBL, USA) at 37℃ for 15 h. LGG was used as a commercial probiotic strain. *Escherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 15313, *Salmonella* Typhimurium P99, and *Staphylococcus aureus* KCCM 11335 as food-borne pathogenic bacteria were grown in tryptic soy broth (TSB; Becton-Dickinson, USA) at 37℃ for 24 h.

Hemolytic activity

L. plantarum G72 and LGG were streaked onto Columbia agar containing 5% (w/v) sheep blood and cultured at 37℃ for 24 h. *L. plantarum* G72, LGG, and food-borne pathogenic bacteria were expressed for signs of β-hemolysis (clear zones around colonies), α-hemolysis (green-hued zones around colonies) or γ-hemolysis (no clear zones around colonies), respectively.

Antimicrobial activity

L. plantarum G72 was assessed for antimicrobial activity against four food-borne pathogenic bacteria (*L. monocytogenes* ATCC 15313, *S.* Typhimurium P99, *E. coli* ATCC 25922, and *S. aureus* KCCM 11335) with modifications [7, 8]. To identify the antimicrobial activity, 3 μl of *Lactobacillus* strains was spotted on MRS agar and incubated at 37℃ for 24 h. Then, 4 ml of TSB soft agar containing 100 μl of the indicator food-borne pathogenic bacteria (10^6 CFU/ml) was overlaid and incubated at 37℃ for 24 h. The diameters (mm) of the clear zones were measured.

Inhibition of adherence of food-borne pathogenic bacteria to HT-29 cells

Inhibitory activity of adherence of food-borne pathogenic bacteria was determined with a modified deferred method reported in Jeon *et al*. [9]. HT-29 cells (1×10^5 cells/well) were seeded into 24-well plates and incubated at 37℃ for 24 h. About 10^6 CFU/well of food-borne pathogenic bacteria with or without *L. plantarum* G72 were added and incubated at 37℃ for 2 h. Plates were washed twice with phosphate buffered saline (HyClone, USA) to remove non-adherent cells. To detach HT-29 cells, 1 ml of 1% (v/v) Triton X-100 solution added to 24-well plates. The mixture was spread on Oxford (Becton-Dickinson), xylose lysine desoxycholat (XLD; Becton-Dickinson), eosine methylene blue (EMB; Becton-Dickinson), and mannitol salt agar (MSA; Becton-Dickinson) for *E. coli*, *L. monocytogenes*, *S.* Typhimurium, and *S. aureus*, respectively, to measure viable cells.

Production of folate

To identify the production of folate, *L. plantarum* G72 and LGG were cultured on MRS broth at 37℃ for 15 h. The *Lactobacillus* strains were centrifuged at 14,240 × *g* for 10 min and the supernatants were filtered through a membrane filter (0.45 μm). Production of folate was identified using folic acid ELISA kit (Cell Biolabs INC, USA).

Short-chain fatty acids analysis

Sample preparation. To analyze the production of short-chain fatty acids (SCFAs), *Lactobacillus* strains were cultured on MRS broth containing 2% arabinose at

37℃ for 15 h. The *Lactobacillus* strains were centrifuged at 14,240 × *g* for 10 min and the supernatants were filtered through a membrane filter (0.2 μm).

HPLC analysis. HPLC was measured using Agilent 1100 system (Agilent Technologies, USA). The column was a reverse phase with Hypersil GOLD aQ column (4.6 mm × 150 mm, 5 μm) (Thermo Fisher Scientific, USA). The elution was used with 50 mM potassium phosphate buffer (pH 2.8) with a 1.25 ml/min flow rate and 20 μl of injection volume. UV absorbance was measured at 210 nm. The standards of SCFAs including lactic acid, acetic acid, propionic acid, and butyric acid were purchased from Sigma-Aldrich (Germany). All samples were filtered through a membrane filter (0.2 μm) and then HPLC was measured.

Statistical analysis

All experiments were conducted in triplicates and are presented as the mean ± standard deviation. Significant differences among the means were determined by one-way analysis of variance (ANOVA) and Duncan's multiple range test. Significant difference between two groups was determined by Student's t-test (SPSS software version 19; IBM, USA). The values were considered statistically significant for $p < 0.05$.

Results and Discussion

Hemolytic activity

The hemolytic activity is known for a safety aspect for the requirement of probiotic strains [23]. Plates were tested for hemolysis sign, which is expressed α-hemolysis (green zones), β-hemolysis (clear zones), and γ-hemolysis (no-clear zones). However, *S. aureus* KCCM 11335 indicated α-hemolysis and *S.* Typhimurium P99 and *E. coli* ATCC 25922 indicated β-hemolysis. Therefore, *L. plantarum* G72 should be non-pathogenic and indicated as safe organism for human health in Table 1.

Table 1. Hemolytic activity of probiotic and food-borne pathogenic bacteria.

Microorganisms	Hemolytic activity[1)]		
	α-Hemolysis	β-Hemolysis	γ-Hemolysis
Lactic acid bacteria			
L. rhamnosus GG	-	-	-
L. plantarum G72	-	-	-
Food-borne pathogenic bacteria			
E. coli ATCC 25922	-	+	-
L. monocytogenes ATCC 15313	-	-	+
S. Typhimurium P99	+	-	-
S. aureus KCCM 11335	-	+	-

[1)]+, positive; -, negative.

Table 2. Antimicrobial activity of *L. rhamnosus* GG and *L. plantarum* G72 against food-borne pathogenic bacteria by deferred method.

Food-borne pathogenic bacteria	Inhibitory diameter (mm)	
	L. rhamnosus GG	*L. plantarum* G72
Escherichia coli ATCC 25922	13.33 ± 0.58^{Aa}	12.00 ± 2.83^{Ab}
Listeria monocytogenes ATCC 15313	15.00 ± 0.00^{Aa}	12.00 ± 2.00^{Ab}
Salmonella Typhimurium P99	2.33 ± 0.58^{Bb}	13.67 ± 1.15^{Aab}
Staphylococcus aureus KCCM 11335	15.00 ± 1.73^{Aa}	17.00 ± 2.65^{Aa}

All values are mean ± standard deviation.
[A-B]The superscript upper-case letters in the same row indicate statistical differences by Student's t-test ($p < 0.05$).
[a-c]The superscript lower-case letters in the same column indicate statistical differences by ANOVA ($p < 0.05$).

Antimicrobial activity

The antimicrobial activity of *L. plantarum* G72 was showed in Table 2. *L. plantarum* G72 exhibited distinct antimicrobial activity against *E. coli* ATCC 25922, *L. monocytogenes* ATCC 15313, *S.* Typhimurium P99, and *S. aureus* KCCM 11335. *S. aureus* KCCM 11335 demonstrated the largest inhibition zone (17 mm). One study demonstrated that *Lactobacillus* sp. (LBS 8) isolated dairy samples showed the highest inhibition zone (14 mm) against *S. aureus* [24]. *L. plantarum* DM69 also exhibited a clear zone of more than 15 mm against food-borne pathogenic bacteria [25]. In addition, various researches have reported the antimicrobial activity of *Lactobacillus* sp. against *Salmonella* sp. Another study has reported the antimicrobial activity of *Enterococcus durans* against *L. monocytogenes*, *E. coli*, and *S.* Typhimurium [23]. The antimicrobial activity is due to the production of metabolites such as organic acids, hydrogen peroxide, bacteriocin, and other compounds that have inhibitory properties [23, 24].

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Table 3. Inhibition activity of *L. plantarum* G72 against adherence of food-borne pathogenic bacteria to HT-29 cells.

Food-borne pathogenic bacteria	Adherent cell no. (Log CFU/ml)	
	Food-borne pathogenic bacteria	Food-borne pathogenic bacteria with *L. plantarum* G72
Escherichia coli ATCC 25922	6.74 ± 0.24^{Ab}	6.57 ± 0.14^{Ab}
Listeria monocytogenes ATCC 15313	4.78 ± 0.11^{Bc}	5.45 ± 0.21^{Ac}
Salmonella Typhimurium P99	6.61 ± 0.15^{Ab}	4.89 ± 0.36^{Bd}
Staphylococcus aureus KCCM 11335	7.25 ± 0.05^{Aa}	6.86 ± 0.14^{Ba}

All values are mean ± standard deviation.
[A-B]The superscript upper-case letters in the same row indicate statistical differences by Student's t-test ($p < 0.05$).
[a-d]The superscript lower-case letters in the same column indicate statistical differences by ANOVA ($p < 0.05$).

Table 4. Production of folate by *L. rhamnosus* GG and *L. plantarum* G72.

	Samples		
	Folate	*L. rhamnosus* GG	*L. plantarum* G72
Production of folate (ng/ml)	80.21 ± 2.34^{a}	50.1 ± 1.26^{c}	55.5 ± 0.21^{b}

All values are mean ± standard deviation.
[a-c]The superscript letters in the same row indicate statistical differences ($p < 0.05$).

Inhibition of adherence of food-borne pathogenic bacteria to HT-29 cells

Adhesion ability of *L. plantarum* G72 to HT-29 cells was assessed by counting the number of food-borne pathogenic bacteria adhered to HT-29 cells in Table 3. *L. plantarum* G72 inhibited the adherence of food-borne pathogenic bacteria to HT-29, the cell numbers of food-borne pathogenic bacteria ranging from 4.89 to 6.86 Log CFU/ml. *L. plantarum* G72 inhibited the adherence of food-borne pathogenic bacteria to HT-29 and food-borne pathogenic bacteria showed a decrease compared to non-treatment of *L. plantarum* G72. The results showed food-borne pathogenic bacteria ranging from 4.89 to 6.86 CFU/ml. The adherence of *E. coli* ATCC 25922 and *S. aureus* KCCM 11335 decreased 0.17 Log CFU/ml and 0.39 Log CFU/ml, especially, the adherence of *S.* Typhimurium P99 decreased remarkably (1.72 Log CFU/ml). However, *L. monocytogenes* ATCC 15313 increased 0.67 Log CFU/ml, although antimicrobial activity of *L. plantarum* G72 against *L. monocytogenes* showed in Table 2. This result was influenced by physical factors such as auto-aggregation, co-aggregation, and hydrophobicity. Additionally, inhibition of adherence of *S.* Typhimurium P99 in the presence *L. brevis* KU15006 to HT-29 cells with an 85.18% reduction in cell number has been reported [7]. *Weissella cibaria* JW15 exhibited a 55.43–69.44% inhibition in adhesion of food-borne pathogenic bacteria [22]. In a previous study, *Lactococcus lactis* KC24, isolated from kimchi, decreased the adhesion of *L. monocytogenes* and *S. aureus* to Caco-2 cells [26]. Therefore, *L. plantarum* G72 could also inhibit the adherence of food-borne pathogenic bacteria to the GI tract.

Production of folate

Production of folate was measured using a minor method with folic acid ELISA kit. Folate is one of the most important components for proper functioning of our body. Folate cannot be synthesized by the human body and has to be obtained through dietary supplements and fortified foods [15, 27]. Production of folate by *L. plantarum* G72 and LGG showed a 50.1 ng/ml and 55.55 ng/ml in Table 4. When compared to 2 strains, it was confirmed that the production of folate showed little difference. Many studies have reported that lactic acid bacteria strains can produce folates [15]. One research indicated that *L. sakei* strains (CRL 2209 and 2210) produced higher folate (730–1,484 ng/g) [15]. *L. rhamnosus* GG IFM4, among the isolated strains, determined that production of folate showed the highest level (35 ng/ml) [28]. In addition, *Lactobacillus fermentum* 8.2 and *L. plantarum* 6.2 isolated from fermented food showed the highest production levels of folate (97 and 93 ng/ml) [29]. Generally, *Lactobacillus* sp. cannot produce folate, however, *L. plantarum*, *L. lactis*, and *Streptococcus thermophilus* are regarded as good functional substances for production of folate [14]. These results seem to show that *L. plantarum* G72 could be used as health food substance in our body.

Short-chain fatty acids analysis

SCFAs are of vital importance in the food industry to improve food quality and safety [30]. *L. plantarum* G72 produced SCFAs such as formic acid, lactic acid, acetic

Table 5. Production of short-chain fatty acids (SCFAs) by *L. rhamnosus* GG and *L. plantarum* G72 in MRS broth.

Microorganisms	Short-chain fatty acids (mg/kg)				
	Formic acid	Lactic acid	Acetic acid	Propionic acid	Butyric acid
L. rhamnosus GG	1,319.29 ± 75.06^{Bc}	10,026.64 ± 264.66^{Ba}	743.66 ± 98.88^{Bd}	2,001.57 ± 201.61^{Ab}	126.73 ± 98.92^{Be}
L. plantarum G72	2,162.20 ± 122.40^{Ab}	11,176.73 ± 531.63^{Aa}	1,380.70 ± 75.65^{Ac}	82.81 ± 7.94^{Bd}	340.99 ± 3.20^{Ad}

All values are mean ± standard deviation.
$^{A-B}$The superscript upper-case letters in the same column indicate statistical differences by Student's t-test ($p < 0.05$).
$^{a-e}$The superscript lower-case letters in the same row indicate statistical differences by ANOVA ($p < 0.05$).

acid, propionic acid, and butyric acid in MRS broth which was detected through the HPLC chromatography in Table 5. SCFAs play an important role in the gastrointestinal condition to protect against food-borne pathogenic bacteria [31]. In this study, SCFAs including formic acid, lactic acid, acetic acid, propionic acid, and butyric acid were produced in the range 82.81 mg/kg to 11,176.73 mg/kg. Especially, lactic acid was the most abundant SCFA produced by *L. plantarum* G72 and LGG (11,176.73 mg/kg and 10,026.64 mg/kg) ($p < 0.05$). One study indicated that the production of lactic acid by *L. plantarum* and *Lactobacillus acidophilus* was 509.40 mg/l and 515.09 mg/l, respectively [30]. It has been demonstrated that *L. plantarum* produced 6.08 g/l of lactic acid [32]. *W. cibaria* JW15 produced 12.60 g/l of lactic acid similar to our results [22]. This study demonstrated that the production of butyric acid by *L. plantarum* (340.99 mg/kg) was higher than that of LGG (126.73 mg/kg). The acid of intestinal bacteria could convert sugar, amino acid, and alcohols to propionic acid or butyric acid [33]. A previous study has reported that butyric acid exhibits some therapeutic effects in intestinal diseases and colon cancer [34]. Therefore, *L. plantarum* G72 might help to improve the gastrointestinal condition by producing SCFAs.

In this study, *L. plantarum* G72 isolated from kimchi demonstrated potential probiotic strain through antimicrobial activity, inhibition of adherence of food-borne pathogenic bacteria to HT-29 cells, production of folate, and production of SCFAs for pregnant women. We indicated that *L. plantarum* G72 should be used as safe probiotic strains by confirming no hemolysis effect, high antimicrobial activity, and inhibition of adherence of food-borne pathogenic bacteria to HT-29 cells as safe organism for human health. In addition, *L. plantarum* G72 could be helped for human as functional effects including production of folate and SCFAs. Therefore, *L. plantarum* G72 may be useful as a potential probiotic and can be applied in health industry.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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