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RESEARCH PAPER

Anti-obesity Potential of *Lactobacillus* spp. Isolated from Infant Feces

Hyemin Kim, Yulah Jeong, Ji-Eun Kim, YongGyong Kim, Nam-Soo Paek, and Chang-Ho Kang

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Abstract Obesity is fast becoming a worldwide health concern and causes a chronic state of oxidative stress and low-grade inflammation, which could be major risk factors for a number of chronic diseases. We investigated the potential of the 61 probiotic candidates isolated from infant feces towards activity of lipid accumulation inhibition, and then we extended the study to evaluate their antidiabetic and antioxidant activity. Seven strains were finally selected based on their ability to inhibit lipid accumulation (> 60%) and productivity yield ($> 1 \times 10^{11}$ colony forming units [CFU]/g): *Lactobacillus acidophilus* MG4558; *Lactobacillus paracasei* MG4592; *Lactobacillus plantarum* MG4553 and MG4555; and *Lactobacillus rhamnosus* MG4502, MG4511, and MG4505. Selected strains showed high α-glucosidase-inhibiting activity (> 50%), except for MG4511 (19.8%), and similar antioxidant activities were reflected by the results of DPPH (21.6-27.5%) and ABTS (40.9-44.9%) assays. With respect to their functional properties as probiotics, most of the strains were resistant to simulated gastric (pH 3 and 4) and intestinal (pH 7 and 8) fluids, and possessed various probiotic-related factors, including auto-aggregation ability, antibiotic susceptibility, enzyme production, and biochemical profiles, and were non-hemolytic. We conclude that our strains may be good probiotic candidates for functional food ingredients to prevent metabolic disorders such as obesity and diabetes.

Keywords: anti-obesity, antidiabetic, antioxidant, probiotics, *Lactobacillus*

Hyemin Kim, Yulah Jeong, Ji-Eun Kim, YongGyong Kim, Nam-Soo Paek, Chang-Ho Kang*
MEDIOGEN Co., Ltd., Jecheon 27159, Korea
Tel: +82-43-842-1940; Fax: +82-43-842-1944
E-mail: changho-kang@naver.com

1. Introduction

According to World Health Organization (WHO), overweight and obesity are defined as abnormal or excessive fat accumulation. These conditions cause chronic oxidative stress and low-grade inflammation, which could be major risk factors for a number of chronic diseases, including diabetes, cardiovascular diseases, and cancer (https://www.who.int/topics/obesity/en/, accessed May, 2020). The prevalence rates of obesity and overweight are increasing worldwide, regardless of age.

The gut microbiome is one of the most important factors regulating metabolic disorders. The gut microbiome affects the host metabolism due to the various enzymes, nutrients, and immune signals expressed by the microbial constituents in the gut, and the gut microbial community changes with the host's lifestyle and can be affected by health disorders [1-3]. Thus, the composition of the gut microbial community can be an indicator of the host's health [4,5].

Probiotics, which are defined as live microorganisms that when administered in adequate amounts can confer a health benefit to the host, can provide various beneficial effects including regulation the adipose tissue in overweight adults and animal models of obesity [6-10]. Since they help maintain the balance of the host's intestinal microbiota, probiotics have been receiving increased scientific attention [11]. Probiotic strains isolated from the human body tend to have beneficial species-specific effects and could be used in humans if they have the properties required to confer beneficial effects (*e.g.*, resistant to acids and bile, proliferate on and adhere to human intestinal cells) in their host [12].

This study aimed to identify probiotic candidates with anti-adipogenic activity among a pool of isolates from infant feces and was extended to explore and evaluate the

antidiabetic and antioxidant effects and probiotic properties of the selected strains.

2. Materials and Methods

2.1. Materials

3T3-L1 preadipocyte cells were purchased from the Korean Cell Line Bank (Korea). Dulbecco's Modified Eagle's Medium (DMEM), bovine calf serum (BCS), and penicillin/streptomycin were purchased from GibcoBRL (USA). Pepsin, pancreatin, p-nitrophenyl α-D-glucopyranoside (PNPG), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS), 3-isobutyl-1-methylxanthine, dexamethasone, and insulin, which were used in the MDI cocktail, were purchased from Sigma-Aldrich (USA). 2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH) was purchased from Alfa Aesar (USA). API 50CHL and API ZYM kits were purchased from BioMérieux (France).

2.2. Isolation and culturing of probiotic strains from infant feces

A total of 61 probiotic candidates, which were isolated from infant fecal samples, were supplied by MEDIOGEN Co., Ltd. (Jecheon, Korea; Table 1). The fecal samples were collected from infants (2-15 months old). A portion of the fresh feces (1 g) was inoculated into 9 mL of 0.85% NaCl and mixed well using a Stomacher sample mixer (3M, Korea) for 3 min, and then the mixture was filtered through Whatman No. 2 filter paper. A 1 mL aliquot of the filtered sample was serially diluted (up to 10^8), and the sample was spread on raffinose-bifidobacterium (RB), neomycin, paromomycin, nalidixic acid, and lithium chloride (NPNL) or Rogosa (a lactobacillus selective agar) agar medium. The plates were incubated at 37°C for 24 h. After incubation, suspected lactic acid bacterial colonies were selected and were streak-purified twice on the same media. The purified colonies were then streaked on bromo cresol purple (BCP) agar to confirm their identity as lactic acid-producing bacteria based on the formation of a yellow zone. The strains were maintained on de Man, Rogosa and Sharpe (MRS) agar medium (Difco, USA) and stored in 20% glycerol at -80°C until use. For culture, the strains were inoculated in MRS broth or agar medium and incubated for 12-18 h at 37°C.

2.3. Identification of probiotic strains

The selected pure colonies were identified using 16S rRNA gene sequencing, which was performed by SolGent Co. (Korea) using universal rRNA gene primers (27F and 1492R). The sequences were compared with available DNA sequences registered at the National Center for Biotechnology Information database (NCBI, http://www.ncbi.nlm.nih.gov/) using Basic Local Alignment Search Tool (BLAST).

2.4. Preparation of cell-free extracts of the isolates

To obtain a cell free extract (CFE), the isolates were lysed by sonicating the microbial cultures using an ultrasonic processor (Korprotech, Korea). Briefly, the isolates were harvested using centrifugation (3,470 × *g* for 5 min at 4°C) and washed three times with phosphate-buffered saline (PBS; pH 7.4) to remove the remaining MRS broth. The cell pellets were then resuspended in PBS at concentrations of 10 mg/mL and lysed using three cycles of sonication at 150 W for 30 s, with 30 s pauses between cycles. To prepare the CFE, the lysed isolates were filtered using a 0.2 μm syringe filter (Advantech, USA), and the filtrate was stored at -80°C until use.

2.5. 3T3-L1 adipocyte differentiation and treatment

3T3-L1 cells were cultured in DMEM supplemented with 10% bovine calf serum (BCS) and 1% penicillin/streptomycin. The cells were passaged every two days to maintain an appropriate number of cells.

To induce adipocyte differentiation, 3T3-L1 cells were seeded in 6-well plates containing DMEM at 1×10^5 cells/mL and cultured for at least two days after the cells reached confluence. The medium was then replaced with DMEM-MDI (containing 0.5 mM IBMX, 1 μM DEX, and 1 μg/mL insulin). On days 2 and 4, the cells were switched to a medium containing only 1 μg/mL insulin. On days 0, 2, and 4, the cells were treated with 0.1 mg/mL of the CFE along with induction medium. Then, the medium was replaced every 2 days for 8 days to induce differentiation [13].

2.6. Evaluation of lipid accumulation by Oil Red O staining

Total lipid accumulation in differentiated 3T3-L1 adipocytes was measured using Oil Red O staining [14]. Briefly, the medium was removed from the cultured 3T3-L1 cells, and the cells were washed twice with PBS (pH 7.4). Then, the cells were fixed by adding 1 mL of 3.7% formaldehyde to each well and incubating for 15 min. Next, the formaldehyde was removed, and the cells were washed three times with PBS. Oil Red O dye solution was prepared by mixing it with distilled water at a ratio of 6:4 and filtering the mixture through a 0.2 μm filter. An aliquot of the prepared Oil Red O solution (500 μL) was added to each well and incubated at room temperature for 30 min and then washed with PBS three times. The cells were observed under a microscope. Finally, Oil Red O staining was quantified by extracting the dye with 100% isopropanol and measuring the absorbance at 540 nm using a microplate reader. The lipid accumulation in the differentiated 3T3-L1 adipocytes

Table 1. Inhibitory effect of lipid accumulation inhibition of probiotic strains isolated from infant fecal samples

Strains		Inhibition lipid accumulation (%)
Bifidobacterium animalis subsp. *lactis*	MG4589	-42.8 ± 1.1
Bifidobacterium breve	MG4528	-31.3 ± 20.6
Lactobacillus acidophilus	MG4558 (This study)	-60.4 ± 11.4
	MG4559	-36.6 ± 8.9
	MG4571	-8.8 ± 4.5
	MG4573	45.0 ± 4.2
Lactobacillus casei	MG4584	-87.3 ± 6.2
Lactobacillus fermentum	MG4500	-40.1 ± 6.8
	MG4510	-58.8 ± 14.8
	MG4529	-52.9 ± 7.2
	MG4530	-56.5 ± 1.4
	MG4531	18.2 ± 16.3
	MG4532	-9.8 ± 7.2
	MG4533	-36.2 ± 3.2
	MG4534	-43.1 ± 9.1
	MG4535	-41.5 ± 0.5
	MG4536	-40.3 ± 0.1
	MG4538	-39.4 ± 1.6
	MG4539	-41.7 ± 2.5
	MG4540	-48.8 ± 1.8
	MG4542	-21.7 ± 15.2
	MG4543	-21.2 ± 19.7
	MG4544	-16.9 ± 14.5
	MG4545	-59.1 ± 3.2
Lactobacillus gasseri	MG4513	12.1 ± 6.0
	MG4514	20.8 ± 1.8
	MG4515	31.9 ± 7.0
	MG4520	-2.8 ± 3.0
	MG4521	16.4 ± 11.5
	MG4523	29.4 ± 11.5
	MG4524	-70.3 ± 6.8
	MG4503	-8.3 ± 4.1
	MG4506	2.8 ± 6.1
	MG4507	-4.3 ± 5.1
	MG4508	-9.0 ± 4.4
	MG4512	1.7 ± 2.2
	MG4575	-46.1 ± 3.9
	MG4582	-72.9 ± 3.1
	MG4583	-53.6 ± 4.0
	MG4588	- 99.0 ± 6.2
	MG4594	7.7 ± 13.8
Lactobacillus paracasei	MG4577	32.2 ± 9.6
	MG4592 (This study)	-77.3 ± 3.3
Lactobacillus plantarum	MG4519	-48.4 ± 11.4
	MG4537	-79.6 ± 0.8
	MG4553 (This study)	-84.1 ± 5.3
	MG4554	-92.5 ± 1.4
	MG4555 (This study)	-79.3 ± 4.6
	MG4556	-25.5 ± 45.7
	MG4557	-29.1 ± 18.4
	MG4585	-93.2 ± 3.3
	MG4586	-54.3 ± 10.7
	MG4587	-80.0 ± 0.7
	MG4591	-58.0 ± 1.0
Lactobacillus rhamnosus	MG4501	-74.5 ± 7.5
	MG4502 (This study)	-97.3 ± 4.0
	MG4511 (This study)	-77.5 ± 7.8
	MG4505 (This study)	-85.1 ± 7.0
Lactobacillus salivarius	MG4525	35.4 ± 3.7
	MG4526	-44.3 ± 10.4
	MG4527	-2.0 ± 12.5

- value represents a inhibition rate of lipid accumulation; + value represents a acceleration rate of lipid accumulation.

was calculated using the following formula:

$$\text{Lipid accumulation (\%)} = A_s/A_c \times 100 \quad (1)$$

where A_s is the absorbance of the MDI with sample and A_c is the absorbance of the MDI without sample at 540 nm.

2.7. α-Glucosidase inhibiting activity assay

To investigate the antidiabetic effects of the *Lactobacillus* spp. strains, inhibition of α-glucosidase was analyzed using a modified version of the method described by Chen *et al*. [15]. Briefly, 25 μL of CFS was added to a reaction mixture containing 150 μL of 0.01 M PBS (pH 7.0) and 75 μL of 0.02 M p-nitrophenyl a-D-glucopyranoside (PNPG) solution and pre-incubated at 37ºC for 10 min. The reaction was initiated by adding 50 μL of α-glucosidase (0.17 units/mL), and the mixture was incubated at 37ºC for 10 min. The reaction was terminated by adding 1 mL of 0.1 M Na_2CO_3, and the amount of ρ-nitrophenol (PNP) released was determined by measuring the absorbance at 405 nm. The percent inhibition was calculated using the following equation:

$$\text{Inhibition (\%)} = [1 - (C - D)/(A - B)] \times 100 \quad (2)$$

in which A is the absorbance of α-glucosidase alone, B is the absorbance without α-glucosidase or the sample, C is the absorbance of α-glucosidase and sample, and D is the absorbance of the sample alone.

2.8. Antioxidant activity

The DPPH radical scavenging assay was performed according to the method of Blois [16] with a slight modification. Briefly, the strain was mixed with a 0.05 mM DPPH solution (1:2) and adjusted to an OD_{600} of 1.0. Then, the mixture was incubated at room temperature for 30 min in the dark. Controls were prepared by mixing ethanol with DPPH solution. The supernatant was obtained by centrifugation at 13,000 rpm for 1 min, and then the absorbance was measured at 517 nm. Each sample was assayed in triplicate. The results were compared with ascorbic acid (100 μg/mL), and scavenging activity was calculated using the following equation:

$$\text{Scavenging effect (\%)} = (A_c - A_s)/A_c \times 100 \quad (3)$$

in which A_s is the absorbance of the test sample and A_c is the absorbance of the control at 517 nm.

ABTS scavenging activity was measured according to the method of Re [17], with slight modifications. Briefly, radical cation was prepared by mixing 7 mM ABTS with 2.45 mM potassium persulfate (1:1, v/v) and incubating at room temperature in the dark for 24 h. Then, the strains were adjusted to OD_{600} of 1.0 by mixing with ABTS solution (1:2) and incubated for 10 min at room temperature in the dark. The reaction mixture was centrifuged at 13,000 rpm for 1 min, and the absorbance of the supernatant was measured at 734 nm. Each assay was performed in triplicate. The results were compared with ascorbic acid (100 μg/mL), and the activity was calculated using the following equation:

$$\text{Scavenging rate (\%)} = (A_c - A_s)/A_c \times 100 \quad (4)$$

in which A_s is the absorbance of the test sample and A_c is the absorbance of the control at 734 nm.

2.9. Survival under simulated human gastrointestinal tract conditions

To simulate human gastric juice, a low pH solution was prepared according to the method described by Maragkoudakis [18]. Briefly, cells harvested by centrifugation (3,750 × *g* for 5 min at 4°C) after culturing for 18 h were washed twice with PBS (pH 7.4). Then, the washed cells (10^8 CFU/mL) were resuspended in the simulated gastrointestinal solution, including simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). SGF was prepared using MRS broth (adjusted to pH 3 and 4 with 1 N HCl) containing 3 g/L of pepsin. SIF was prepared by mixing pancreatin (1 g/L) with sterilized PBS (adjusted to pH 7 and 8 with 1 N NaOH).

To test for pepsin and pancreatin resistance, *Lactobacillus* spp. strains were suspended into SGF and SIF, respectively, and incubated at 37°C for 3 h. The resistance of the strains was evaluated by counting viable cells on MRS agar plates and is reported CFU/mL.

2.10. Assessment of enzyme production and biochemical profile characterization

To measure the enzymatic activities and carbohydrate utilization of four selected strains, the bacteria were incubated on an MRS agar plate for 18 h at 37°C. Then, colonies were selected for assay using API ZYM and API 50 CHL kits according to the manufacturer's instructions (BioMérieux, France). Enzyme activity was evaluated on a scale of 0 (no activity) to 5 (≥ 40 nM of product released) at 10 nM intervals based on the API ZYM color reaction chart.

2.11. Auto-aggregation assay

Auto-aggregation assays were performed according to the method of Kos *et al*. [19], with slight modifications. *Lactobacillus* spp. strains were grown in MRS medium for 18 h at 37ºC. Then, the cells were harvested by centrifugation at 3,750 × *g* for 5 min, washed three times with PBS (pH 7.0), and resuspended in PBS to an OD_{600} of 1.0. The cell suspensions (4 mL) were mixed by vortexing for 10 s and incubated at room temperature for 5 h. Then, the absorbance of a 0.1 mL aliquot of the suspension was measured at

600 nm in an EPOCH 2 Microplate reader (BioTek, USA). Auto-aggregation (%) was calculated using the following equation:

$$\text{Auto-aggregation (\%)} = (1 - (A_5/A_0)) \times 100 \quad (5)$$

in which A_5 is the absorbance at 5 h of incubation and A_0 is the absorbance at time zero.

2.12. Antibiotic susceptibility assay

Antibiotic susceptibility was evaluated according to EFSA guidelines using the MIC test strip method European Food Safety Authority (EFSA) guidelines [20]. The susceptibility of the bacterial strains to nine antimicrobial agents was tested: ampicillin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, streptomycin, tetracycline, and vancomycin. Bacterial strains were grown in MRS medium for 18 h at 37°C and then harvested by centrifugation at 3,750 × *g* for 5 min, washed three times with PBS (pH 7.0), and resuspended in PBS to a 0.5 McFarland standard. The cell suspensions were inoculated onto Brain Heart Infusion agar (BHI; Difco, USA) with swabs, and the plates were allowed to dry for 10-15 min. Then, MIC test strips (Liofilchem, Italy) were placed on the agar surface according to the manufacturer's recommendations. The plates were incubated at 37°C for 20 h, and the results were read.

2.13. Hemolytic activity

To assay hemolytic activity, the strains were streaked onto Tryptic Soy Agar (TSA; Difco, USA) medium containing 5% sheep blood and incubated at 37°C for 48 h. After incubation, the plates were observed for the formation of a clear zone (β-hemolysis), a greenish zone (α-hemolysis), or no zone (γ-hemolysis, non-hemolytic) around the colonies.

2.14. Observing bacterial cells through field emission scanning electron microscope (FE-SEM)

The morphological characteristics of the selected cells were determined by field emission scanning electron microscopy (FE-SEM) (Hitachi, S-4300SE, Japan) performed at Inha University.

2.15. Statistical analysis

All experiments were performed in triplicate, and the results are presented as the mean ± standard deviation (SD). Statistical analyses were performed using the R (version 3.6.2) and PRIMER-E software packages.

3. Results and Discussion

Obesity induces lipotoxicity and oxidant stress and causes an insulin resistance state, which then creates hyperglycemia with compensated hepatic gluconeogenesis. In addition, obesity causes perturbation of the intestinal microbial composition, known as dysbiosis [21]. Several studies have revealed the associations between intestinal dysbiosis and chronic low-grade inflammation and metabolic disorders like obesity and diabetes [22-24]. Consequently, probiotics have been receiving increased attention to maintain a healthy condition or prevent specific symptoms. To address this current interest, we tried to obtain and identify probiotics with enhanced anti-obesity, antidiabetic, and antioxidant functionality to apply to food supplements.

3.1. Anti-obesity activity and selection of the isolated strains

The 61 isolates were identified based on the 16S rRNA gene sequencing results. The candidate strains were from 2 genera and 10 species: *Bifidobacterium animalis* subsp. *lactis*, *Bifidobacterium breve*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus fermentum*, *Lactobacillus gasseri*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, and *Lactobacillus salivarius* (Table 1).

To select anti-obesity functional probiotic strains, the lipid accumulation-inhibiting effects of all 61 isolates in differentiated 3T3-L1 adipocytes were evaluated by measuring the intensity of Oil Red O staining. The inhibitory rates against lipid accumulation of the candidates showed strain specificity and were found to vary (-45-108%; Table 1). 3T3-L1 cells treated with MDI formed many more lipid droplets than untreated cells (control). Among the isolates, strains with high lipid accumulation inhibition abilities (> 50%) were selected (n = 25), and these strains were evaluated for fermented product yield (> 1×10^{11} CFU/g; data not shown). From the screening strategies, seven strains were selected: *L. acidophilus* MG4558; *L. paracasei* MG4592; *L. plantarum* MG4553 and MG4555; and *L. rhamnosus* MG4502, MG4511, and MG4505 ($p < 0.001$; Table 1).

Previous studies have shown that *Lactobacillus* spp. decreased lipid accumulation. For example, 1:1 mixture of *L. plantarum* KY1032 and *Lactobacillus curvatus* HY7601 caused an 18% decrease in lipid accumulation in 3T3-L1 cells [25]. Moreover, *L. plantarum* K10 inhibited lipid accumulation of 32.6% in 3T3-L1 [26]. Our results corresponded with these studies, as we demonstrated several *Lactobacillus* spp. had an excellent anti-obesity effect by suppressing lipid accumulation.

3.2. Anti-diabetic activities of the selected strains

To identify the strains with excellent hypoglycemic ability, the effect of the selected seven strains on the α-glucosidase

activity was examined (Fig. 1A). Most strains showed relatively high α-glucosidase inhibition and the inhibitory activities in descending order were as follows; *L. plantarum* MG4555 (91.6%), *L. acidophilus* MG4558 (90.3%), *L. paracasei* MG4592 (81.7%), *L. rhamnosus* MG4502 (63.4%), *L. plantarum* MG4553 (62.1%), *L. rhamnosus* MG4505 (57.5%), and *L. rhamnosus* MG4511 (19.8%).

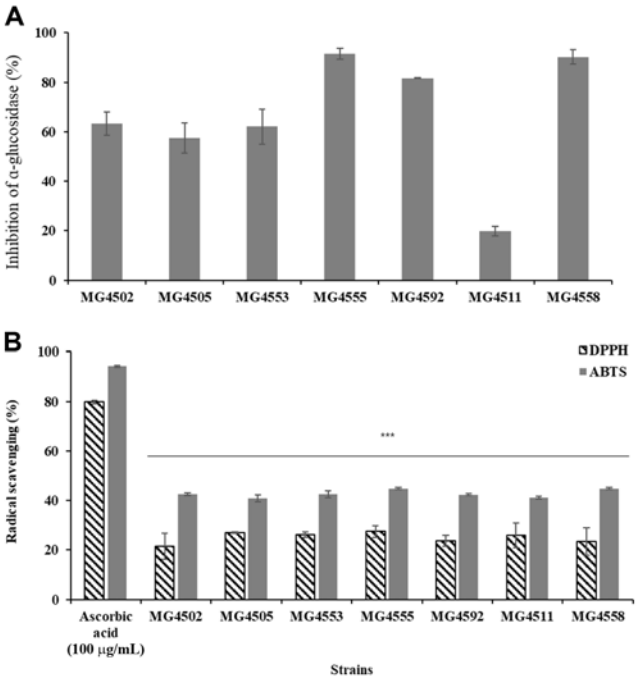

Fig. 1. α-Glucosidase-inhibiting activities (A) and antioxidant activities (B) of the seven candidate probiotic *Lactobacillus sp.* strains. All values are presented as the mean ± SD of three independent experiments. Significant correlations between ascorbic acid and cell treatment are shown by asterisks ($p < 0.001$).

The selected strains showed high performance of inhibition of lipid accumulation and α-glucosidase activity (> 50%) except for *L. rhamnosus* MG4511 (19.8%). α-Glucosidase digests dietary starch and degrades oligosaccharides to glucose, leading to a rapid increase in postprandial glucose. Thus, α-glucosidase inhibitors function as oral antidiabetic agents and are used for the treatment of type 2 diabetes (T2D). Other observatory experiments reported that oral administration of probiotics helped to prevent and/or alleviate diabetes via various mechanisms, such as enhancing insulin sensitivity, alleviating impaired glucose tolerance, regulating lipid metabolism, improving antioxidant status, and modulating the composition of the microbial flora and short chain fatty acid contents of the gut [27-29]. Further studies will be needed to demonstrate the mode of action in *in vivo* study.

3.3. Antioxidant activities of selected strains

The antioxidant activities of the selected strains were evaluated using DPPH and ABTS radical scavenging assays. The DPPH free radical scavenging activities of the probiotic strains ranged from 21.6% to 27.5%, and the ABTS radical scavenging activities ranged from 40.9% to 44.9% (Fig. 1B). Overall, the selected strains showed similar O_2 radical scavenging activities.

The seven strains were investigated as to their reactive oxygen scavenging ability. All strains showed a higher level of radical scavenging ability in the ABTS assays than in the DPPH assays. This might be due to the inherent characteristics of ABTS (hydrophilic) and DPPH (hydro-

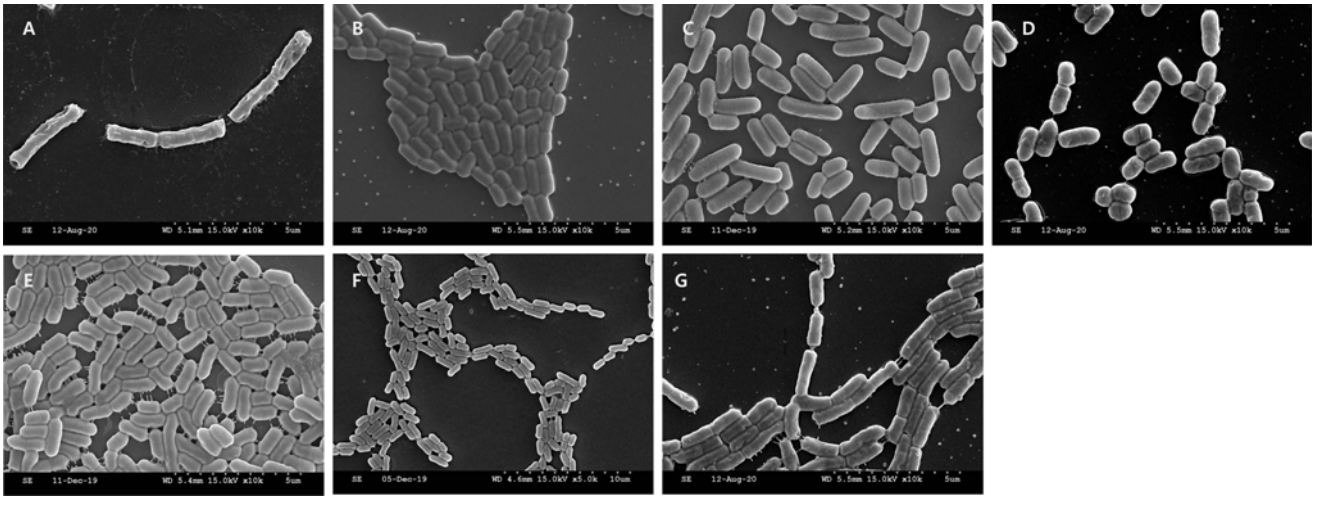

Fig. 2. Scanning electron microscopy images of the selected strains. *Lactobacillus acidophilus* MG4558 (A), *Lactobacillus paracasei* MG4592 (B), *Lactobacillus plantarum* MG4553 (C), *L. plantarum* MG4555 (D), *Lactobacillus rhamnosus* MG4502 (E), *L. rhamnosus* MG4505 (F), and *L. rhamnosus* MG4511 (G). All strains were observed at × 10,000 magnification, except for *L. rhamnosus* MG4505 at × 5,000 magnification.

phobic) assays [30]. Our results corresponded with those of other studies on the antioxidant activities of lactobacilli; Li *et al*. reported the antioxidant activities of *L. plantarum* strains derived from food [31], and Afify *et al*. reported the ABTS radical scavenging activities of *Lactobacillus reuteri* and *Lactobacillus breve* [32]. The antioxidative efficacy of probiotics is considered to be related to by-products of cells [33,34]. Several studies have shown that exopolysaccharides (EPS) from probiotic bacterium act as antioxidants [35,36]. Our most strains, especially *L. rhamnosus* strains, showed the cells were linked by strands of EPS (Fig. 2). Most probiotics including *Lactobacillus* are known to be the most prominent EPS producing bacteria [37,38]. Further studies will be needed to demonstrate the mode of action in *in vivo* study.

3.4. Correlation analysis of the anti-obesity, anti-diabetic, and antioxidant activities of the probiotic strains

To determine the relationships between the various *in vitro* activities of the strains, *i.e*., the correlations among the DPPH and ABTS scavenging activities, α-glucosidase inhibition, and lipid accumulation inhibition were evaluated by multiple mean comparisons and Pearson correlation analysis (Table 2). ABTS scavenging activity was significantly correlated with lipid accumulation inhibition ($p < 0.01$), and α-glucosidase inhibition was significantly correlated with lipid accumulation inhibition ($p < 0.05$). However, there were no statistically significant correlations between DPPH scavenging activities and the other analyzed characteristics ($p > 0.05$).

As we predicted, the lipid accumulation inhibition activity and α-glucosidase activity of the strains were significantly correlated ($p < 0.05$; Table 2). Our results indicated that the selected strains might contribute to the amelioration in co-morbid condition of obesity and T2D, termed as diabesity. Also, the antioxidant activities were significantly correlated with lipid accumulation inhibition ($p < 0.01$; Table 2). Perhaps, our selected strains may contribute to prevent T2D by inhibiting lipid accumulation with antioxidant ability.

3.5. Probiotic properties of selected strains

The selected strains were exposed to simulated gastric fluid conditions (pH 3 and 4) and simulated intestinal conditions (pH 7 and 8). All strains survived under the simulated gastrointestinal conditions (Table 3); cell viability ranged from 5.5 to 8.6 log CFU/mL under gastric conditions and from 7.8 to 8.6 log CFU/mL under intestinal conditions (Table 3). The adherence abilities of the selected strains were assessed by measuring auto-aggregation at 5 h (Table 4). *L. acidophilus* MG4558 showed strong auto-aggregation (94.3%) and aggregated immediately, forming a precipitate and a clear solution. The other strains showed lower auto-aggregation abilities (40-62%), and the suspensions formed precipitates but remained turbid (Table 4).

Table 2. The relationship between the activities of the candidate probiotic isolates

	DPPH[a]	ABTS	α-Glucosidase inhibition
DPPH			
ABTS	0.27		
α-Glucosidase inhibition	0.10	-0.12	
Lipid accumulation inhibition	0.01	0.56**	0.54*†

[a]The values shown in the table are Pearson correlation coefficients. Significant correlations are shown in bold and marked with asterisks. *, $p < 0.05$; **, $p < 0.01$
†Value was calculated without results from *Lactobacillus rhamnosus* MG4511.

Table 3. Survival of selected bacterial strains under simulated gastrointestinal conditions

Strains	Simulated gastric fluid[1)]		Simulated intestinal fluid[2)]	
	pH 3	pH 4	pH 7	pH 8
Lactobacillus acidophilus MG4558	6.3 ± 0.1	5.5 ± 0.1	8.3 ± 0.03	8.3 ± 0.03
Lactobacillus paracasei MG4592	7.9 ± 0.01	8.0 ± 0.1	7.9 ± 0.01	8.0 ± 0.03
Lactobacillus plantarum MG4553	7.8 ± 0.002	7.8 ± 0.01	7.8 ± 0.02	7.8 ± 0.03
Lactobacillus plantarum MG4555	7.7 ± 0.02	7.8 ± 0.01	7.8 ± 0.02	7.8 ± 0.03
Lactobacillus rhamnosus MG4502	8.6 ± 0.03	8.6 ± 0.1	8.6 ± 0.1	8.5 ± 0.03
Lactobacillus rhamnosus MG4511	8.6 ± 0.01	8.6 ± 0.03	8.6 ± 0.1	8.6 ± 0.1
Lactobacillus rhamnosus MG4505	8.5 ± 0.1	8.6 ± 0.1	8.6 ± 0.01	8.6 ± 0.02

The results are expressed as the mean ± SD, and each value is the mean of three replicate measurements from three independent experiments.
[1)]Simulated gastric tolerance results are shown as the viable counts (log CFU/mL) for each strain after incubation at pH 3 and 4 for 3 h at 37°C.
[2)]Simulated intestinal tolerance results are shown as viable counts (log CFU/mL) for each strain after incubation at pH 7 and 8 for 3 h at 37°C.

Table 4. Auto-aggregation ability of the selected *Lactobacillus* spp. isolates

Strains	Auto-aggregation (%)
Lactobacillus acidophilus MG4558	94.3 ± 0.6
Lactobacillus paracasei MG4592	40.2 ± 3.9
Lactobacillus plantarum MG4553	62.0 ± 1.2
Lactobacillus plantarum MG4555	56.3 ± 2.2
Lactobacillus rhamnosus MG4502	34.0 ± 3.6
Lactobacillus rhamnosus MG4511	41.1 ± 4.2
Lactobacillus rhamnosus MG4505	49.7 ± 3.1

Results are presented as mean ± SD from three independent experiments.

Probiotics must be evaluated for the production of certain enzymes to ensure that they do not produce potentially toxic substances. Therefore, the enzymatic activities of the probiotic strains were assessed using the API ZYM system (Table 5). Generally, all strains showed high leucine arylamidase activity, but no β-glucuronidase, α-mannosidase, or trypsin activity. Among the selected strains, *L. rhamnosus* MG4502, MG4511, and MG4505 showed relatively higher alkaline phosphatase, lipase (C14), α-chymotrypsin, α-galactosidase, and α-fucosidase activities than the other strains. Carbohydrate utilization was assessed using the API 50 CHL system, and the results are summarized in Table 6. All the selected strains were able to use the following carbohydrates: D-galactose, D-glucose, D-fructose, D-mannose, N-acetyl-glucosamine, amygdalin, arbutin, esculin, salicin, D-cellobiose, D-trehalose, D-melezitose, and gentiobiose. However, all the strains were not able to use glycerol, erythritol, L-xylose, D-adonitol, methyl-β D-xylopyranoside, methyl-α D-glucoside, inulin, D-raffinose, starch, glycogen, xylitol, D-lyxose, D-fucose, D-arabitol, 2-keto-gluconate, and 5-keto-gluconate.

The antibiotic resistance of seven probiotic strains was assessed by determining the minimal inhibitory concentrations (MIC) of nine antibiotics and the epidemiological cut-off values as suggested by EFSA guidelines [20]. Most strains were sensitive to ampicillin, gentamicin, streptomycin, erythromycin, and clindamycin except for *L. acidophilus* MG4558, which was resistant to gentamicin, kanamycin, and chloramphenicol (Table 7). Hemolysis is a major virulence factor of pathogenic bacteria. All tested *Lactobacillus* spp. strains displayed γ-hemolysis (*i.e.*, were non-hemolytic) at 48 h of incubation on blood agar plates (data not shown). And the FE-SEM photomicrographs showed the appearance of the selected strains (Fig. 2). All strains were rod-shaped and especially *L. rhamnosus* cells (MG4502, MG4505, and MG4511) were linked by strands of exopolysaccharide (Fig. 2E-G).

Qualification of probiotics requires evaluation of various attributes, including adhesion to and colonization of mucosal and epithelial cells, acid and bile salt tolerance, probiotic stability, and viability. Before a strain can be used as a probiotic, it is essential to evaluate its probiotic functions and technical specifications as well as its safety. According to the results from this study, our selected seven strains showed stability and safety in various assessments. Some strains have resistance to a few antibiotics, and antibiotic resistance is thought to be advantageous for the survival of probiotic microorganisms in the gastrointestinal tract during antibiotic treatment, since killing a probiotic strain by an antibiotic either ingested for therapeutic purposes or present in food would decrease its functionality. Thus,

Table 5. Enzymatic activities of selected strains as measured using the API ZYM system

Enzyme	MG4558	MG4592	MG4553	MG4555	MG4502	MG4511	MG4505
Alkaline phosphatase	0	0	0	0	2	1	1
Esterase (C4)	1	2	0	0	3	4	3
Esterase lipase (C8)	0	2	0	0	3	4	4
Lipase (C14)	0	0	0	0	2	2	2
Leucine arylamidase	4	5	5	5	5	5	5
Valine arylamidase	0	5	4	4	5	5	5
Cystine arylamidase	1	0	0	0	3	3	3
α-Chymotrypsin	0	0	0	0	1	2	2
Acid phosphatase	1	1	1	1	3	3	3
Naphthol-AS-BI-phosphohydrolase	3	2	1	1	5	4	3
α-Galactosidase	0	0	0	0	2	1	1
β-Galactosidase	3	0	2	2	3	3	3
α-Glucosidase	0	3	2	2	2	1	1
β-Glucosidase	0	0	3	3	5	5	5
N-Acetyl-β-glucosaminidase	0	0	3	3	0	0	0
α-Fucosidase	0	0	0	0	3	4	4

All strains were negative for trypsin, β-glucuronidase, and α-mannosidase.

Table 6. Summary of the carbohydrate fermentation profiles of the selected probiotic candidate strains as measured using the API 50 CHL system

Substrate	MG4558	MG4592	MG4553	MG4555	MG4502	MG4511	MG4505
D-Arabinose	-	-	-	-	+	+	+
L-Arabinose	-	-	+	+	-	-	-
D-Ribose	-	+	+	+	-	+	+
D-Xylose	+	-	-	-	-	-	-
L-Sorbose	-	+	-	-	-	-	-
L-Rhamnose	-	-	+	+	-	-	-
Dulcitol	-	-	-	-	+	+	+
Inositol	-	-	-	-	+	+	+
D-Mannitol	-	+	+	+	+	+	+
D-Sorbitol	-	+	+	+	+	+	+
Methyl-α D-Mannoside	-	-	+	+	-	-	-
D-Maltose	+	+	+	+	-	-	-
D-Lactose	+	-	+	+	-	-	-
D-Melibiose	-	-	+	+	-	-	-
D-Sucrose	+	+	+	+	-	-	-
D-Turanose	-	+	+	+	-	-	-
D-Tagatose	-	+	-	-	+	+	+
L-Fucose	-	-	-	-	+	+	+
L-Arabitol	-	+	-	-	-	-	-
Gluconate	-	+	-	-	+	+	+

+, the strain can ferment the substrate; -, the strain cannot ferment the substrate.
All strains fermented D-galactose, D-glucose, D-fructose, D-mannose, N-acetyl-glucosamine, amygdalin, arbutin, esculin, salicin, D-cellobiose, D-trehalose, D-melezitose, and gentiobiose. All strains did not ferment glycerol, erythritol, L-xylose, D-adonitol, methyl-β D-xylopyranoside, methyl-α D-glucoside, inulin, D-raffinose, starch, glycogen, xylitol, D-lyxose, D-fucose, D-arabitol, 2-keto-gluconate, and 5-keto-gluconate.

Table 7. Minimum inhibitory concentrations (MICs) of various antibiotics for the selected probiotic strains

Antimicrobials	MG4558	MG4592	MG4553	MG4555	MG4502	MG4511	MG4505
Ampicillin	S	S	S	S	S	S	S
Gentamicin	R	S	S	S	S	S	S
Kanamycin	R	R	S	S	S	S	S
Streptomycin	S	S	n.r.	n.r.	S	S	S
Tetracycline	S	S	R	R	S	S	S
Chloramphenicol	R	R	R	S	S	R	S
Erythromycin	S	S	S	S	S	S	S
Vancomycin	S	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
Clindamycin	S	S	S	S	S	S	S

Antibiotic susceptibility was determined according to EFSA guidelines [20]. The inhibitory zone was measured, and susceptibility was determined according to the standard index. S: Susceptible, a probiotic strain was defined as susceptible when it was inhibited at a concentration equal to or lower than the established cut-off value; R: Resistant, a probiotic strain was defined as resistant when it was not inhibited at a concentration higher than the established cut-off value. n.r., not required.

resistance to antibiotics is recognized as a particularly important probiotic feature [39].

4. Conclusions

This study aimed to select superior probiotic strains with high anti-obesity, anti-diabetic, and antioxidant activities, by evaluating the characteristics of *Lactobacillus* spp. strains isolated from infant feces. In our strains, there was a significant correlation between lipid accumulation-inhibiting and α-glucosidase-inhibiting activities. Our seven selected probiotic candidates have demonstrated probiotic functions, along with high stability under simulated gastrointestinal conditions and safety. Taken together, our data indicate that these probiotic strains could be useful resources for probiotic development. Further studies are needed to elucidate their mode of action, establish how they modulate the gut microbiome, and determine their efficacy in *in vivo* studies.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Statement

Neither ethical approval nor informed consent was required for this study.

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