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RESEARCH PAPER

Antioxidant and Probiotic Properties of Lactobacilli and Bifidobacteria of Human Origins

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Abstract Oxidative stress can cause various diseases including inflammation, neurological disorders, cancer, diabetes, and cardiovascular diseases. Due to the current search for natural antioxidants, probiotics have received increasing scientific interest and are facing a growing industrial demand. Although various strains of lactobacilli and bifidobacteria are currently used in numerous health food supplements, their antioxidative activities have been relatively poorly identified. Therefore, in this work, we evaluated the *in vitro* effect of antioxidative activities (through assays of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) radical scavenging) and probiotic functional properties (cell viability in a simulated gastrointestinal tract, enzyme production, carbohydrate availability, and safety assessments) of *Lactobacillus* spp. and *Bifidobacterium* spp. isolated from human origins. From the nitric oxide (NO) assay screening, four strains (*Bifidobacterium animalis* subsp. *lactis* MG741, *B. breve* MG729, *L. reuteri* MG505, and *L. rhamnosus* MG316) were selected based on the yield amount of ferment productivity (> × 10^{10} CFU/g) and showed high antioxidant activities ranging from 22.2% to 38.2% in DPPH free radical scavenging, and 50.0% to 93.6% in ABTS radical scavenging. Regarding their functional properties as probiotics, these four strains were resistant to simulated gastric (pH 3 and 4) and intestinal fluids (pH 7 and 8), and showed potential for the promotion of health based on hemolysis, auto-aggregation, antibiotic susceptibility, enzyme production, and biochemical profiles. Altogether, our results showed that the selected probiotic strains may be good candidates as food ingredients to mitigate oxidative stress-related symptoms.

Keywords: nitric oxide, antioxidant, probiotics, *Lactobacillus*, *Bifidobacterium*

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1. Introduction

Oxidative stress is caused by excessive reactive oxygen species (ROS) and can cause damage to lipids and proteins, as well as DNA [1]. Although most living organisms can deal with oxidative stress through intrinsic antioxidant defense mechanisms such as enzymatic and non-enzymatic processes or repair systems, the imbalance between ROS and these antioxidant systems could lead to cell and tissue damage [2,3]. An increase in oxidative stress is closely related to various diseases including hypertension [4,5], atherosclerosis [6], hyperlipidemia [7], diabetes [8], cancer [9], rheumatoid arthritis [10], Parkinson's disease [11], and aging [12].

Antioxidants are widely used as health food supplements for promoting good health and preventing diseases. Recently, an interest in natural sources or alternative medicines from bio-resources has grown considering the lack of safety of artificial compounds [13,14]. Along with the increasing scientific interest in natural antioxidants, numerous studies have reported antioxidative activities of various natural sources such as food, plants, mushrooms, and microbes [15-19]. In this context, probiotics have received increasing interest by scientists in the search for naturally sourced antioxidants [20-29]. Probiotics may modulate the host's defenses against oxidative stress through antioxidant activities, the regulation of signaling pathways, metal ion chelation, and modulation of intestinal microflora [21]. According to

the *in vitro* and *in vivo* studies, probiotic bacteria show strain-specific differences in antioxidative properties [30,31]. Probiotic strains were shown to limit excessive amounts of reactive radicals *in vivo* by promoting intestinal saccharolytic metabolism and to reduce doxorubicin-induced oxidative stress [30]. Although various strains of lactobacilli and bifidobacteria have been used in health foods, their antioxidative activities and associated mechanisms have been relatively understudied [2].

Microorganisms show strong relationships with their typical habitat and origin. Strains isolated from the human body have a beneficial effect of species-specific interactions and could be considered for use in humans if they have a beneficial effect on their host [32]. Probiotics of human origin colonize the human gut and adhere to intestinal cells, where they confer resistance to acid and bile, produce antimicrobial substances, and protect against cariogenic and pathogenic bacteria. In addition, probiotic microorganisms isolated from humans are safe for human consumption and have clinically validated health effects [33].

This preliminary study aimed to investigate the *in vitro* antioxidant and probiotic properties of lactobacilli and bifidobacteria isolated from human origins to determine whether these microorganisms could be applied as a functional food.

2. Materials and Methods

2.1. Probiotic strains and culture conditions

A total of 112 probiotic strains were supplied by MEDIOGEN Co., Ltd. (Jecheon, Korea) [34]. The strains were isolated from various origins such as human feces, breast milk, and fermented food. The strains were identified as 16 species belonging to the *Lactobacillus*, *Lactococcus*, *Bifidobacterium*, and *Streptococcus* genera (Table 1). Cultures were used from frozen stocks stored at −80°C in MRS broth (de Man, Rogosa and Sharpe, Difco, USA) containing 15–25% glycerol. All strains (inoculum 2%, v/v) were cultured and maintained in MRS broth at 37°C, except for *Streptococcus thermophilus*, which was able to grow at 42°C.

2.2. NO production screening

RAW 264.7 macrophage cells were purchased from the Korean Cell Line Bank (KCLB). These cells were grown at 37°C and 5% CO_2 in fully humidified air and sub-cultured every 3 days to a confluence of 95%. For routine sub-cultures, Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) was supplemented with 10% fetal bovine serum (FBS, Gibco), penicillin (100 units/mL), and streptomycin (100 μg/mL).

NO formation was detected by the accumulation of

Table 1. Inhibitory activity of isolated lactic acid bacteria on nitric oxide (NO) production by lipopolysaccharide (LPS)-induced RAW 264.7 macrophages

Origin	MG strains	Inhibition rate (%)[1]
Breast milk	*L. reuteri* MG505 (in this study)	77.5 ± 4.4
	L. gasseri MG4503	65.0 ± 4.9
	L. gasseri MG4506	44.8 ± 0.2
	L. gasseri MG4508	50.3 ± 3.8
	L. gasseri MG4512	65.0 ± 1.4
Human	*L. plantarum* MG989	60.8 ± 46.6
	L. plantarum MG4215	91.0 ± 18.4
	L. plantarum MG4221	86.5 ± 49.3
	L. plantarum MG4234	59.0 ± 50.6
	L. gasseri MG4243	64.8 ± 30.6
	L. fermentum MG4244	97.4 ± 28.2
	L. fermentum MG4254	90.3 ± 44.6
	L. fermentum MG4258	52.5 ± 73.8
	L. fermentum MG4261	65.8 ± 17.8
	L. plantarum MG4270	27.6 ± 49.2
	L. rhamnosus MG4288	22.2 ± 11.9
	L. rhamnosus MG4289	6.9 ± 27.6
	L. rhamnosus MG4298	30.4 ± 25.0
	L. fermentum MG4231	81.4 ± 1.3
	L. paracasei MG4272	42.9 ± 15.5
	L. plantarum MG4229	44.2 ± 10.1
	L. plantarum MG4296	36.7 ± 6.2
Infant feces	*B. bifidum* MG731	70.8 ± 11.7
	B. breve MG729 (in this study)	80.4 ± 2.0
	L. rhamnosus MG316 (in this study)	74.8 ± 43.5
	B. animalis subsp. *lactis* MG741 (in this study)	92.8 ± 30.5
	L. fermentum MG4532	63.5 ± 1.5
	L. fermentum MG4534	40.0 ± 13.8
	L. plantarum MG4553	62.4 ± 32.4
	L. plantarum MG4555	34.1 ± 5.3
	L. plantarum MG4556	10.8 ± 12.4
	L. plantarum MG4557	80.5 ± 5.2
	L. plantarum MG4519	50.9 ± 5.7
	L. plantarum MG4537	39.7 ± 17.4
	L. fermentum MG4530	- 23.3 ± 92.3
	L. fermentum MG4531	58.1 ± 9.4
	L. fermentum MG4535	30.0 ± 15.0
	L. fermentum MG4536	33.0 ± 1.5
	L. fermentum MG4538	81.5 ± 5.0
	L. fermentum MG4539	- 63.4 ± 5.0
	L. fermentum MG4540	45.4 ± 5.8
	L. fermentum MG4542-1	48.5 ± 13.4
	L. fermentum MG4545-1	3.5 ± 6.2
	L. gasseri MG4520	- 55.2 ± 4.3
	L. gasseri MG4521	58.0 ± 17.8
	L. gasseri MG4524	48.3 ± 45.9
	L. acidophilus MG4558	49.7 ± 22.0
	L. acidophilus MG4559	55.9 ± 2.9

Table 1. Continued

Origin	MG strains	Inhibition rate (%)[1]
Infant feces	*L. gasseri* MG4513	11.2 ± 2.9
	L. gasseri MG4514	56.6 ± 62.7
	L. fermentum MG4542-2	42.9 ± 11.5
	L. salivarius MG4527	51.9 ± 31.0
	L. salivarius MG4525	74.7 ± 21.3
	L. fermentum MG4543	67.5 ± 15.5
	L. fermentum MG4544	26.0 ± 38.5
	L. fermentum MG4545-2	49.4 ± 63.2
	L. acidophilus MG4573	34.6 ± 37.4
Fermented food	*L. delbrueckii* subsp. *bulgaricus* MG5166	48.3 ± 2.4
	L. acidophilus MG5228	66.8 ± 4.4
	L. casei MG311	67.5 ± 7.3
	L. casei subsp. *paracasei* MG310	47.2 ± 3.4
	S. thermophilus MG5140	47.9 ± 0.5
	L. delbrueckii MG515	63.8 ± 2.0
	L. salivarius MG5212	85.7 ± 2.4
	L. plantarum MG5254	71.4 ± 4.5
	L. plantarum MG5270	47.7 ± 2.2
	L. plantarum MG5287	74.6 ± 19.6
	L. plantarum MG5289	78.7 ± 18.4
	L. plantarum MG5324	70.0 ± 25.1
	L. fermentum MG5154	30.7 ± 3.9
	L. plantarum MG5143	72.1 ± 12.8
	L. plantarum MG5155	54.4 ± 20.9
	L. plantarum MG5197	28.6 ± 1.3
	L. plantarum MG5203	83.3 ± 5.0
	L. plantarum MG5239	87.1 ± 3.5
	L. fermentum MG5341	37.9 ± 8.6
	L. paracasei MG5135	- 122.5 ± 7.3
	L. paracasei MG5178	- 37.9 ± 16.4
	L. paracasei MG5189	- 113.2 ± 8.6
	L. paracasei MG5219	- 78.9 ± 5.5
	L. paracasei MG5310	- 22.0 ± 6.2
	L. casei MG5275	- 115.9 ± 1.8
	L. casei MG5296	- 94.7 ± 4.6
	L. acidophilus MG5339	50.3 ± 3.8
	L. acidophilus MG5340	76.9 ± 7.3
	Lac. lactis MG5125	69.9 ± 9.8
	Lac. lactis MG5126	67.1 ± 34.7
	Lac. lactis MG5127	77.6 ± 44.2
	Lac. lactis MG5128	13.0 ± 46.9
	Lac. lactis MG5129	71.4 ± 8.8
	Lac. lactis MG5278	40.3 ± 26.7
	S. thermophilus MG5152	60.4 ± 66.1
	S. thermophilus MG5299	- 82.5 ± 19.8
	S. thermophilus MG5300	63.0 ± 34.5
	S. thermophilus MG5301	76.0 ± 54.1
	S. thermophilus MG5303	101.9 ± 9.4
	S. thermophilus MG5304	95.5 ± 65.1
	S. thermophilus MG5342	3.2 ± 19.0
	S. thermophilus MG5343	26.0 ± 35.2
Fermented food	*S. thermophilus* MG5344	42.2 ± 39.3
	S. thermophilus MG5345	- 28.6 ± 25.1
	S. thermophilus MG5150	- 76.0 ± 23.2
	L. helveticus MG5161	13.0 ± 27.7
	L. helveticus MG5162	- 16.7 ± 3.3
	L. helveticus MG5220	- 16.7 ± 1.7
	L. helveticus MG5290	- 15.0 ± 17.4
	L. delbrueckii subsp. *bulgaricus* MG5164	57.7 ± 13.1
	L. delbrueckii subsp. *bulgaricus* MG5165	- 90.2 ± 39.5
	L. delbrueckii subsp. *bulgaricus* MG5167	2.8 ± 10.0
	L. delbrueckii subsp. *bulgaricus* MG5168	- 37.8 ± 7.6
	L. delbrueckii subsp. *bulgaricus* MG5169	- 54.9 ± 3.2
	L. delbrueckii subsp. *bulgaricus* MG5170	- 197.6 ± 17.1

[1]means ± SD (n = 3).
B., *Bifidobacterium*; *E.*, *Enterococcus*; *L.*, *Lactobacillus*; *Lac.*, *Lactococcus*; *S.*, *Streptococcus*

nitrite, an indicator of NO synthesis, in culture medium via the Griess reaction [35]. RAW 264.7 cells were plated at 2×10^5 cells/well in a 96-well plate and stimulated with 1 μg/mL lipopolysaccharide (LPS), followed by the addition of the isolated bacterial strains (10^7 cells/well). After 24 h of incubation, NO concentration was determined by measuring the amount of nitrite in the cell culture supernatant using the Griess reagent. An absorbance measurement at 550 nm was obtained using the Epoch 2 microplate reader (BioTek, USA). Fresh culture medium was used as the blank control for all experiments.

According to the results of the NO assay, the probiotic strains showed a wide range of NO production inhibition rates (Table 1). This result indicated that bacterial strains would have different functional characteristics, even if they belong to the same species. Among the 112 probiotic strains, there were no significant correlations between the efficacy of NO production inhibition and the fermentation production yield ($P > 0.05$; data not shown). Thus, we simply selected four strains from human origins according to the best yield amount of fermentation product ($> \times 10^{10}$ CFU/g) for application in the food industry: *Lactobacillus reuteri* MG505, *L. rhamnosus* MG316, *Bifidobacterium animalis* subsp. *lactis* MG741, and *Bifidobacterium breve* MG729. The other candidates, which showed a high NO production inhibition rate but low production yield, require culture optimization to improve their value as a biological resource.

2.3. Antioxidant activity of lactic acid bacteria

2.3.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity

The DPPH radical scavenging assay was performed according to Blois [36] with slight modifications. Briefly, the selected strains adjusted to an OD_{600} of approximately 1.0 with phosphate-buffered saline (PBS, pH 7.4) were added to 0.05 mM DPPH solution (1:2 v/v) and mixed well. Then, the mixtures were left to stand at room temperature for 30 min in the dark. The control reaction was prepared using ethanol added to the DPPH solution. The absorbance of each mixture was quantified at 517 nm. Each sample assay was performed in triplicate. The results were compared with those of ascorbic acid (10 and 100 μg/mL), and the antioxidant activity was calculated using the following formula:

$$\text{Scavenging effect (\%)} = (A_c\text{-}A_s)/A_c \times 100 \quad (1)$$

where A_s is the absorbance of the test sample, and A_c is the absorbance of the control at 517 nm.

2.3.2. 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) radical scavenging activity

Scavenging of the ABTS radical was measured according to Re *et al.* [37]. Briefly, the radical cation was prepared by mixing 7 mM of ABTS with 2.45 mM potassium persulfate (1:1 v/v) and leaving the mixture at room temperature in the dark for 24 h. After incubation, the ABTS solution was further diluted with distilled water (DW) until the initial absorbance value of 0.7 ± 0.005 at 734 nm was reached. Then, 50 μL of the selected strain samples and 100 μL of ABTS solution were mixed and incubated for 10 min at room temperature. The absorbance of the mixture was measured at 734 nm. Each sample assay was performed in triplicate, and the scavenging rate was calculated as follows:

$$\text{Scavenging rate (\%)} = (A_c\text{-}A_s)/A_c \times 100 \quad (2)$$

where A_s is the absorbance of the test sample, and A_c is the absorbance of the control at 734 nm.

2.4. Probiotic properties of strains

2.4.1. Strain survival under conditions simulating the human gastrointestinal tract

To simulate human gastric juice, low pH conditions were induced according to the methods described by Maragkoudakis *et al.* [38]. Briefly, the cells were harvested using centrifugation (3,470×g for 5 min at 4°C) after culture for 18 h and were washed twice with PBS (pH 7.0). The washed cells (10^8 CFU/mL) were resuspended in the simulated gastrointestinal solutions.

To test for resistance to pepsin and pancreatin, bacterial cells were suspended in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF), respectively. SGF was prepared using MRS broth (pH 3 and 4; adjusted with 1 N HCl) with 3 g/L of pepsin (Sigma-Aldrich, USA). SIF was prepared by supplementing sterilized PBS (pH 7 and 8; adjusted with 1 N NaOH) with pancreatin (Sigma-Aldrich) to a final concentration of 1 g/L. Cells were resuspended in SGF and SIF, and incubated at 37°C for 0, 1, 2, 3, or 4 h. The resistance of the selected strains in every condition was evaluated by cell viability using cell counts on MRS agar plates to obtain numbers of colony-forming units per mL (CFU/mL).

2.4.2. Assessment of enzyme production and biochemical profile characterization

To measure enzyme activities and carbohydrate availability, the four selected strains were grown on an MRS agar plate for 18 h at 37 or 42°C. Each of the strains was assayed using API ZYM and API 50 CHL kits with cell colonies according to the manufacturer's instructions (bioMérieux, France). Enzyme activity and degrees of substrate hydrolysis were determined according to the intensity of coloration.

2.4.3. Auto-aggregation

Auto-aggregation assays were performed according to Kos *et al.* [39] with slight modifications. Bacterial cells were grown in MRS medium for 18 h at 37°C. The cells were harvested by centrifugation at 3,470×g for 5 min, washed twice with PBS (pH 7.0), and resuspended in PBS to an optical density at 600 nm (OD_{600}) of 1.0. Cell suspensions (4 mL) were mixed by vortexing for 10 s and incubated at room temperature for 5 h. The OD_{600} of 0.1 mL of the upper suspension was measured with a microplate reader. Auto-aggregation (%) was calculated using the following formula:

$$\text{Auto-aggregation (\%)} = [1\text{-}(A_5/A_0)] \times 100 \quad (3)$$

where A_5 is the absorbance after 5 h of incubation and A_0 is the absorbance at time zero.

2.4.4. Antibiotic susceptibility

Antibiotic susceptibilities were determined according to the European Food Safety Authority (EFSA) guidelines [40]. The selected four strains were assayed for antibiotic susceptibility by the minimum inhibitory concentration (MIC) test strip method. A total of nine antimicrobial agents were tested against bacterial strains. The tested agents were ampicillin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, streptomycin, tetracycline, and vancomycin. Bacteria were grown for 18 h at 37°C in MRS medium. The cells were harvested by centrifugation

at 3,470×g for 5 min, washed twice with PBS (pH 7.0), and resuspended in PBS to a McFarland turbidity of 0.5. The cell suspensions were inoculated on Brain Heart Infusion agar (BHI, Difco) using swabs. Plates were allowed to dry for 10 to 15 min, and MIC Test Strips (Liofilchem, Italy) were placed on the agar surface according to the manufacturer's recommendations. Plates were incubated at 37°C, and results were read 20 h after inoculation.

2.4.5. Hemolytic activity

For evaluating hemolytic activity, the selected strains were grown in MRS for 18 h at 37°C, streaked onto Tryptic Soy Agar (TSA, Difco) medium with 5% sheep blood, and incubated at 37°C for 48 h. Thereafter, the plates were observed for the formation of a clean zone (β-hemolysis), a greenish zone (α-hemolysis), or no such zone (γ-hemolysis) around the colonies.

2.5. Statistical analysis

All experiments were performed in triplicate, and the results are represented as mean ± standard deviation (SD). Statistical analysis was performed to evaluate statistical differences using R (version 3.6.2; The R Foundation for Statistical Computing). Significant differences between the results were evaluated using Tukey's honestly significant difference test.

3. Results and Discussion

3.1. Inhibition of LPS-induced NO production by the potential probiotic strains

NO assay is a competitive assay in which the radical scavenger competes with molecular oxygen to react with the NO radical. In this study, NO assay was used to test selected candidate probiotic strains for anti-inflammatory activity in macrophages (RAW 264.7). As shown in Fig. 1, LPS markedly induced NO production (46.3 ± 1.0 μM) compared to the control (4.1 ± 0.2 μM). After treatment (× 10^7 cells/well), all strains significantly reduced the levels of NO production ($P < 0.001$). Among the tested strains, *B. animalis* subsp. *lactis* MG741 showed the highest NO inhibition (7.1 μM) in LPS-induced cells, followed by *B. breve* MG729 (12.4 μM), *L. reuteri* MG505 (13.6 μM), and *L. rhamnosus* MG316 (42.8 μM): these strains showed inhibition rates of 92.8%, 80.4%, 77.5%, and 74.8%, respectively (Fig. 1; Table 2).

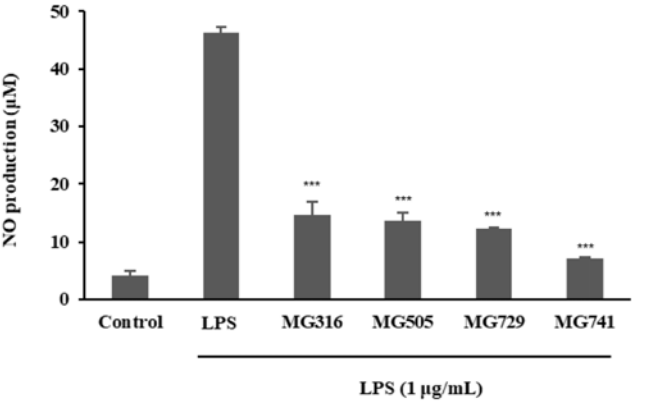

Fig. 1. Inhibition of nitric oxide production in lipopolysaccharide (LPS)-induced RAW 264.7 cells treated with selected strains (10^7 cells/well). Significant correlations between LPS and cell treatment are shown by asterisks ($p < 0.001$).

NO is a reactive nitrogen species that promotes biosynthesis of inflammatory mediators to deepen inflammation. According to Squadrito and Pryor, an important mechanism by which superoxide dismutase mimetics attenuate inflammation is reducing peroxynitrite formation by simply removing superoxide anion before reaction with NO [41]. That is, NO itself does not have cytotoxicity but produces $ONOO^-$, which has strong cytotoxicity, when it reacts with superoxide. Thus, our selected strains may produce potent antioxidants that inhibit NO production. Although the molecular mechanisms of the strains were not elucidated in this study, a strong reduction of NO production indicated that our probiotic strains may affect ROS scavenging in an oxidative stress condition. However, the metabolites related to the inhibition of NO production should be further investigated in an *in vivo* study.

Table 2. Results of the antioxidant and nitric oxide (NO) assays using the selected probiotic strains in this study

Antioxidant assays	DPPH radical scavenging (%)	ABTS radical scavenging (%)	Inhibition of NO production (%)
Ascorbic acid (100 μg/mL)	38.2 ± 1.4	93.3 ± 0.1	-
Ascorbic acid (10 μg/mL)	27.9 ± 2.3	76.7 ± 0.02	-
B. breve MG729	35.4 ± 0.8	93.6 ± 0.2	80.4 ± 2.4
B. animalis subsp. *lactis* MG741	38.2 ± 1.6	86.4 ± 0.2	92.8 ± 2.4
L. reuteri MG505	33.5 ± 0.8	55.9 ± 0.1	77.5 ± 1.4
L. rhamnosus MG316	22.2 ± 2.4	50.0 ± 0.6	74.8 ± 7.3

Results are presented as the means ± standard deviation (SD) from three independent experiments. *B.*, *Bifidobacterium*; *L.*, *Lactobacillus.*

3.2. *In vitro* antioxidant properties of the selected strains

The antioxidant activities of the four selected strains, which showed high inhibition of NO production, were evaluated by DPPH and ABTS radical scavenging activities. The DPPH free radical scavenging activities of the probiotic strains ranged from 22.2% to 38.2% (Table 2). *B. animalis* subsp. *lactis* MG741 showed the highest radical scavenging activity (38.2%) and similar antioxidant activity when compared with an ascorbic acid (100 μg/mL) control (38.2%), followed by *B. breve* MG729 (35.4%), *L. reuteri* MG505 (33.5%), and *L. rhamnosus* MG316 (22.2%). Regarding ABTS radical scavenging activities, results of the strains ranged from 50.0% to 93.6% (Table 2). *B. breve* MG729 showed the highest radical scavenging activity (93.6%), followed by *B. animalis* subsp. *breve* MG741 (86.4%), *L. reuteri* MG505 (55.9%), and *L. rhamnosus* MG316 (50.0%).

All four strains showed high antioxidant activities, indicating that the selected probiotic strains possess the ability to reduce ROS. The trends of antioxidant activity and NO production inhibition rate coincided. Our results corresponded with other studies regarding antioxidant activities of lactobacilli and bifidobacteria [30,42-45]. Li *et al.* [42] reported the antioxidant activities of *Lactobacillus plantarum* strains derived from food, and Afify *et al.* [43] reported the ABTS radical scavenging effects of *L. reuteri* and *L. breve*. Lin and Yen [44] evaluated the inhibitory effect of *Bifidobacterium longum*, and Kim *et al.* [45] isolated antioxidative *Bifidobacterium* species from infant fecal samples. It is known that probiotics produce bioactive compounds with beneficial properties, including antioxidant activity, and may act through specific molecular mechanisms responsible for defense against oxidative stress according to strain specificity [30,46].

3.3. Probiotic properties of selected strains

A main property of probiotics as functional supplements is their tolerance to digestive stress [47,48]. The probiotic bacteria must survive and settle at the epithelium of the intestinal tract. Thus, potential probiotic strains must be screened for functionality and technical specifications in addition to going through a safety assessment.

3.3.1. Survival of the strains under simulated gastrointestinal conditions

The probiotic strains were exposed to simulated gastric fluid conditions (pH 3 and 4) and intestinal conditions (pH 7 and 8). Both simulated gastrointestinal conditions resulted in similar cell viabilities, ranging from 7.8 to 8.0 log CFU/mL for *L. rhamnosus* MG316 and *L. reuteri* MG505 (Table 3). *Bifidobacterium* strains, on the other hand, relatively proliferated in intestinal conditions (7.2–8.3 log CFU/mL) in comparison to gastric conditions (5.6–6.4 log CFU/mL).

All four strains generally endured and survived the gastrointestinal conditions (pH 3, 4, 7, and 8). These results indicated that the selected strains are likely to survive in both the stomach and intestinal juices. *Lactobacillus* spp. were relatively more resistant to gastric conditions than *Bifidobacterium* spp. The acid resistance of lactic acid bacteria has been reported to be related to changes in its glycolytic flux, its ability to control intracellular pH, and a cell membrane ATPase. This suggests that the high resistances of *L. reuteri* MG505 and *L. rhamnosus* MG316 to gastric acid were due to intracellular pH regulation and the action of an ATPase [49]. For *Bifidobacterium* spp., a microencapsulation technology would be a good solution to enhance its survivability in gastric conditions [50].

3.3.2. Auto-aggregation

Auto-aggregation is an important probiotic property in the prevention of surface colonization by pathogens [51], and lactic acid bacteria generally have a variable range of auto-aggregation capacities [52]. The adherence abilities of the four probiotic strains were measured by their auto-aggregation in 5 h. All strains showed percentages of auto-aggregation above 30%, and *L. reuteri* MG505 presented the highest auto-aggregation (64.4%) compared to the other strains (> 30%, Fig. 2). In the present study, our strains showed relatively high auto-aggregation values [53-55].

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

Strains	Simulated gastric fluid[1]		Simulated intestinal fluid[2]	
	pH 3	pH 4	pH 7	pH 8
B. breve MG729	5.6 ± 0.15	6.1 ± 0.02	8.3 ± 0.05	8.3 ± 0.09
B. animalis subsp. *lactis* MG741	6.1 ± 0.06	6.4 ± 0.15	7.2 ± 0.10	7.4 ± 0.03
L. rhamnosus MG316	7.9 ± 0.01	8.0 ± 0.05	8.0 ± 0.03	8.0 ± 0.02
L. reuteri MG505	7.8 ± 0.02	7.8 ± 0.06	7.8 ± 0.02	7.8 ± 0.01

The results are expressed as means ± standard deviation (SD); each data point represents the average of three repeated measurements from three independently replicated experiments. [1]Simulated gastric tolerance results are shown as viable counts (log CFU/mL) for each strain at pH 3 and pH 4 after 3 h. [2]Simulated intestinal tolerance results are shown as viable counts (log CFU/mL) for each strain at 37°C after 4 h. *B.*, *Bifidobacterium*; *L.*, *Lactobacillus*.

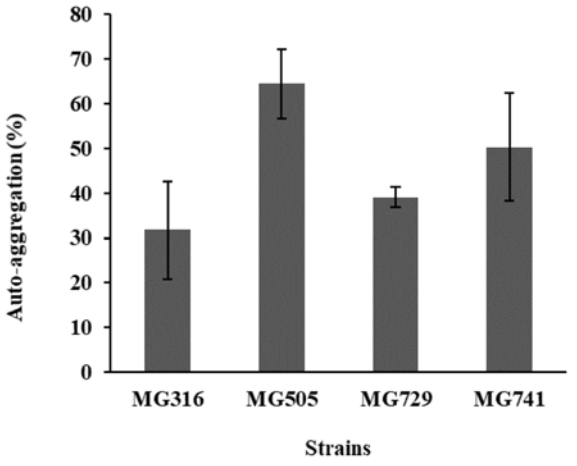

Fig. 2. Evaluation of the auto-aggregation of probiotic strains resuspended in phosphate-buffered saline (PBS, pH 7.0) after 5 h.

3.3.3. Antibiotic susceptibility

The antibiotic resistance of the four probiotic strains was assessed through an MIC test. Results from all four strains were within the epidemiological cut-off values suggested by EFSA [40]. *L. rhamnosus* MG316 was resistant to kanamycin and chloramphenicol, *B. breve* MG729 was resistant to tetracycline, and *B. animalis* subsp. *lactis* MG741 was resistant to gentamicin and streptomycin. All strains were sensitive to ampicillin, erythromycin, and clindamycin (Table 4).

The use of probiotics with antibiotic resistance has caused concern because these strains could carry antibiotic resistance genes and transmit the same to pathogens through horizontal gene transfer. However, when a probiotic strain is killed by antibiotics ingested for therapeutic purposes, its functionality is decreased. The antibiotic resistance of probiotic microorganisms is thought to be advantageous for survival in the gastrointestinal tract during medical treatment with an antibiotic. In this respect, resistance to antibiotics is also recognized as a very important factor [56]. Therefore, probiotics have a double-edged sword effect; they can be useful for individuals with unbalanced intestinal microflora due to the administration of various antibiotics, but they may also transfer resistance genes to other bacteria [57]. In terms of the potential risk of probiotics, the US FDA evaluated the "safety of probiotic use" with "reasonable certainty" [57].

3.3.4. Hemolytic activity

The four probiotic strains exhibited the γ-hemolysis effect after 48 h of incubation on blood agar plates (data not shown). Hemolysis remains the main virulence factor of pathogenic bacteria, and probiotic strains must be safe, especially within the host body.

3.3.5. Assessment of enzyme production and biochemical profile characterization

The enzymatic activity patterns of the probiotic strains were assessed using an API ZYM system (Table 5). Probiotics must be evaluated for the production of appropriate enzymes in order to avoid the production of potentially toxic substances. The selected strains did not produce lipase, β-glucuronidase, N-acetyl-β-glucosaminidase, or α-mannosidase. Among them, β-glucuronidase is a bacterial carcinogenic enzyme that exerts negative effects on the liver [58].

The utilization of carbohydrates, measured with the API 50 CHL system, is summarized in Table 6. None of the selected strains fermented glycerol, erythritol, D-arabinose, D-adonitol, methyl-β D-xylopyranoside, dulcitol, inositol, inulin, xylitol, D-tagatose, D-fucose, L-fucose, D-arabitol, or L-arabitol (Table 6). Probiotics, especially lactic acid bacteria, are able to produce lactic acid using carbohydrates. In this study, the selected strains were able to use the following carbohydrates in common: D-glucose, D-fructose, D-maltose,

Table 4. Minimum inhibitory concentrations (MIC) of different antibiotics for the selected probiotic strains

Antibiotics	*B. breve* MG729	*B. animalis* subsp. *lactis* MG741	*L. rhamnosus* MG316	*L. reuteri* MG505
Ampicillin	S	S	S	S
Gentamicin	S	R	S	S
Kanamycin	n.r.	n.r.	R	S
Streptomycin	S	R	S	S
Tetracycline	R	S	S	S
Chloramphenicol	S	S	R	S
Erythromycin	S	S	S	S
Vancomycin	S	S	n.r.	n.r.
Clindamycin	S	S	S	S

Antibiotic susceptibilities determined according to the European Food Safety Authority (EFSA) guidelines [40]. The inhibitory zone around the antibiotic strips was measured and determined according to the standard index. S: Susceptibility, a probiotic strain was defined as susceptible when inhibited at a specific antimicrobial concentration equal or inferior to the established cut-off value; R: Resistant, a probiotic strain was defined as resistant when not inhibited by a concentration higher than the established cut-off value. n.r., not required. *B.*, *Bifidobacterium*; *L.*, *Lactobacillus*.

Table 5. Enzyme activities of selected strains assayed by the API ZYM system

Enzyme assayed	*B. breve* MG729	*B. animalis* subsp. *lactis* MG741	*L. rhamnosus* MG316	*L. reuteri* MG505
Alkaline phosphatase	2	1	1	1
Esterase (C4)	1	0	0	1
Esterase lipase (C8)	0	0	1	1
Leucine arylamidase	0	4	4	3
Valine arylamidase	0	0	1	2
Crystine arylamidase	0	1	5	0
Trypsin	0	0	1	0
α-Chymotrypsin	0	1	2	0
Acid phosphatase	2	0	1	5
Naphtol-AS-BI-phosphohydrolase	0	4	3	3
α-Galactosidase	4	2	1	0
β-Galactosidase	5	3	0	3
α-Glucosidase	5	2	1	1
β-Glucosidase	4	0	0	0
α-Fucosidase	0	0	1	0

Enzyme activities were classified from 0 (no activity) to 5 (≥ 40 nM of product released) with 10 nM intervals in the API ZYM color reaction chart. All strains showed a negative response to lipase (C14), β-glucuronidase, N-acetyl-β-glucosaminidase, and α-mannosidase. *B.*, *Bifidobacterium*; *L.*, *Lactobacillus*.

Table 6. Summary of carbohydrate fermentation profiles of the selected bacterial strains obtained using the API 50 CHL system

Substrate	MG316	MG505	MG729	MG741	Substrate	MG316	MG505	MG729	MG741
L-arabinose	+	–	–	–	Arbutin	+	–	–	+
D-ribose	+	+	–	+	Esculin	+	–	+	+
D-xylose	–	+	+	+	Salicin	+	–	+	+
L-xylose	–	–	–	+	D-cellobiose	+	–	–	–
D-galactose	+	–	–	–	D-melibiose	+	–	+	+
D-mannose	+	–	+	–	D-trehalose	+	–	–	–
L-sorbose	–	–	+	+	D-melezitose	+	–	–	–
L-rhamnose	–	–	–	+	D-raffinose	–	+	+	+
D-mannitol	+	–	–	–	Starch	–	–	–	+
D-sorbitol	+	–	–	–	Glycogen	–	–	+	–
Methyl-α D-mannoside	+	–	–	–	Gentiobiose	+	–	+	+
Methyl-α D-glucoside	–	–	+	–	D-turanose	+	–	+	–
N-acetyl-glucosamine	+	–	+	+	D-lyxose	–	–	–	+
Amygdalin	+	–	+	–					

+ indicates the ability of the strains to ferment the substrate, and – indicates the inability of the strains to ferment the substrate.
All strains fermented D-glucose, D-fructose, D-maltose, D-lactose, and D-sucrose, and not glycerol, erythritol, D-arabinose, D-adonitol, methyl-β D-xylopyranoside, dulcitol, inositol, inulin, xylitol, D-tagatose, D-fucose, L-fucose, D-arabitol, and L-arabitol.
MG316, *Lactobacillus rhamnosus* MG316; MG505, *Lactobacillus reuteri* MG505; MG729, *Bifidobacterium breve* MG729; MG741, *Bifidobacterium animalis* subsp. *lactis*.

D-lactose, and D-sucrose.

This study was conducted with the aim to select superior probiotic strains with a desired antioxidant activity among 112 strains of probiotic candidates obtained from human origins by evaluating their inhibitory activity on NO production. We selected four probiotic strains (*B. animalis* subsp. *lactis* MG741, *B. breve* MG729, *L. reuteri* MG505, and *L. rhamnosus* MG316) that showed high antioxidant activities and demonstrated probiotic functions with high stability and safety in simulated gastrointestinal conditions. Taken together, our data indicated that these probiotic strains could serve as good resources in the field of probiotic development. Further *in vivo* study would be required to evaluate the effect and safety.

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Conflict of Interest

The authors declare no potential conflict of interest.

Ethical Statement

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

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