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Lactobacillus gasseri MG4247 and *Lacticaseibacillus paracasei* MG4272 and MG4577 Modulate Allergic Inflammatory Response in RAW 264.7 and RBL-2H3 cells

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Abstract
Allergic inflammation refers to a hyperimmune reaction that causes hypersensitivity responses such as hives, itchiness, runny nose, and cough due to specific allergens. Allergic diseases are known to be influenced by the diversity and distribution of intestinal microbiota, and *Lactobacill* is known to relieve allergic symptoms by modulating cytokines secreted by T helper type 1 (Th1)/Th2 cells. This study was designed to investigate the effects of *Lactobacillus gasseri* MG4247 and *Lacticaseibacillus paracasei* MG4272, MG4577, and MG4657 on levels of pro-inflammatory cytokines and proteins associated with allergic symptoms in RAW 264.7 macrophages, and RBL-2H3 mast cells, as well as their probiotic properties. MG4247, MG4272, and MG4577 significantly reduced tumor necrosis factor-α and interleukin (IL)-6 levels in LPS-induced RAW 264.7 macrophages, and markedly decreased IL-4, IL-5, and IL-13 levels and STAT6 phosphorylation in DNP-IgE/HSA sensitized RBL-2H3 mast cells. Furthermore, MG4247, MG4272, and MG4577 tolerated the acidic condition with pepsin and basic condition with bile salt, and showed a high adhesion rate (≥ 73.9%). In safety evaluation, MG4247, MG4272, and MG4577 showed no hemolytic or bile salt hydrolase activity and no cytotoxicity to HT-29 cells (≥ 96.7%). Hence, MG4272, MG4272, and MG4577 can be used as candidate probiotic strains to relieve cytokines associated with allergic inflammation.

Keywords Allergic symptom · Inflammation · Probiotics · Immune response

Introduction

Allergies are inflammatory diseases caused by the disorder of the immune system and hypersensitivity [1]. Allergies are caused by various allergens including food, insects, drugs, and the environment, and manifest as this allergic diseases such as rhinitis, anaphylaxis, asthma, and atopic dermatitis [2]. The prevalence of allergies is over 4 million worldwide, and it is increasing annually, making it a major global health concern [3]. Disruption of the normal immune response results in an imbalance between T helper type 1 (Th1) and T helper type 2 (Th2) cells [4]. In addition, macrophages participate in the immune response during allergic inflammation [5]. Macrophages secrete Th1 cytokines such as tumor necrosis factor α (TNFα), and interleukin (IL)-6, which are pro-inflammatory mediators of inflammation by allergen [5, 6]. Th2 cytokines, including IL-4, IL-5, and IL-13, are produced by the phosphorylation of signal transducer and activator of transcription 6 (STAT6) in mast cells, and sensitize allergens [4, 7]. Antihistamines have been used to treat of allergic symptoms; however, this class of drug is not ideal because of its adverse effects, such as dizziness, fatigue, and drowsiness [8]. Therefore, complementary and alternative treatments with minimal side effects are needed to improve allergic inflammation.

Probiotics, including lactic acid bacteria (LAB), are defined as living microorganisms that provide health benefits to the host [9]. Recently, probiotics have been reported to have various physiological roles and beneficial effects on various diseases such as gastrointestinal disorders, metabolic disorders, and allergic inflammation [10, 11]. Song et al. demonstrated that *Lactiplantibacillus plantarum L67* suppressed the expression of pro-inflammatory cytokines in

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bisphenol A-exposed RBL-2H3 mast cells, and inflammatory factors in cadmium-treated RAW 264.7 macrophages [12]. *Bifidobacterium longum* IM55 and *L. plantarum* IM76 are reliving Th2 cytokine levels in house dust mite allergen and ovalbumin-induced allergic mouse models [13, 14]. Probiotics, including *Lacticaseibacillus paracasei*, *Lactobacillus gasseri Lactobacillus acidophilus*, and *Lacticaseibacillus rhamnosus* GG improve the condition of patients with allergic rhinitis by decreasing Th2 and pro-inflammatory cytokine levels [15, 16]. The anti-allergy response of probiotics must be investigated for further experiments based on animal models or clinical studies; additionally, more rapid screening by in vitro tests can save time and economically, and scientifically establish the beneficial effects of probiotics [17].

In this study, *L. gasseri* MG4247 and *L. paracasei* MG4272, MG4577, and MG4567, a species known to relieve cytokines in rhinitis patients, were investigated in RAW 264.7 macrophages and RBL-2H3 mast cells to evaluate the expression of pro-inflammatory and Th2 cytokines. In addition, LAB, which are thought to suppress allergic symptoms, were assessed for the probiotic properties including stability and safety in HT-29 intestinal epithelial cells.

Materials and Methods

Reagents and Apparatus

All the reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). De Man, Rogosa, and Sharpe (MRS) broth was purchased from BD Biosciences (Franklin Lakes, NJ, USA). RAW 264.7, RBL-2H3, and HT-29 cell lines were obtained from the Korean Cell Line Bank (Seoul, Korea). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin (P/S), and phosphate-buffered saline (PBS) were purchased from Gibco (Gaithersburg, MD, USA). HiGene™ Total RNA Prep Kit (BIOFACT, Daejeon, Korea) was reverse-transcribed to cDNA using the SuperScript™ IV First-Strand Synthesis System (Invitrogen, Waltham, MA USA), and FastStart Essential DNA Green Master (Roche, Basel, Switzerland) was used for investigating mRNA expression. RIPA buffer was obtained from iNtRON (Seongnam-si, Gyeonggi-do, Korea) and contained phosphatase and protease inhibitor cocktails (GenDEPOT, Katy, TX, USA). The Pierce BCA Protein Assay Kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibodies against STAT6, and phosphorylated STAT6 (p-STAT6) were purchased from Cell Signaling Technology (Danvers, MA, USA). Amersham™ Imager 600 (GE Healthcare, Chicago, IL, USA) and WesternBright ECL kit (Advansta, San Jose, CA USA) were used for protein image analysis.

Preparation of Cell-Free Supernatants from LAB (SL)

All the LAB used in this study were obtained from MEDIOGEN (Jecheon, Korea). *L. gasseri* MG4247, and *L. paracasei* MG4272, MG4577, and MG4657 were isolated from Korean individuals. The selected strains were identified by the 16S rRNA gene sequencing (SolGent Co., Ltd. Korea). The DNA sequence was registered in the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST; NCBI Accession number-MG4247: MN069036.1, MG4272: MW947164.1, MG4577: MN833017.1, MG4657: ON025789). Based on BLAST, similarity of strains was confirmed through phylogenetic tree (Fig. S1). A phylogenetic tree was constructed with the neighbor-joining method using MEGA software ver. 10.0 (https://www.megasoftware.net/).

To obtain SL, each LAB cultured in MRS broth at 37 °C for 18 h in an anaerobic chamber were centrifuged (4000 × *g*) for 15 min at 4 °C. The obtained SLs were filtered using a 0.2-μm polytetrafluoroethylene (PTFE) membrane (ADVANTEC, Tokyo, Japan), and stored at −70 °C for the next experiment.

Cell Culture

Raw 264.7 macrophages and HT-29 cells were grown at 37 °C, 5% CO_2 in RPMI medium containing 10% FBS and 1% P/S. RBL-2H3 basophilic leukemia mast cells were grown at 37 °C, 5% CO_2 in Minimum Essential Medium (MEM) containing 15% FBS and 1% P/S. Cells were subcultured at 70–80% confluency.

Cell Viability

Cell viability was measured using the 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay [18]. Cells (3×10^4 cells/well) were seeded in 96 well plates and treated with SL for 24 h. MTT solution (0.1 mg/mL) was added to each well, and it was cultured for 1 h. After incubation, the formazan crystals in each well were dissolved in 150 μL DMSO. Absorbance was measured at 550 nm using a microplate reader.

Preparation of Total RNA

RAW 264.7 macrophages were seeded at 3×10^5 cells/well in 6-well plates and incubated for 24 h. The cells were treated with 100 ng/mL of lipopolysaccharide (LPS; *Escherichia coli* O111:B4) with or without samples diluted to 5% (*v*/*v*) in serum-free media (SFM) for 24 h. RBL-2H3 mast cells were seeded at 3×10^5 cells/well in 6-well plates and treated with 110 ng/mL monoclonal anti-dinitrophenyl antibody (DNP-IgE) for 1 h. The cells were incubated with SL, diluted to

5% (*v*/*v*) in SFM, for 1 h, and then with 25 ng/mL albumin, dinitrophenyl (DNP-HSA) for 1 h. Total RNA of RAW 264.7 and RBL-2H3 cells was isolated using HiGene™ Total RNA Prep Kit following the manufacturer's protocols. Total RNA from RAW 264.7 macrophages and RBL-2H3 mast cells was isolated using the HiGene™ Total RNA Prep Kit.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA from RAW 264.7 macrophages and RBL-2H3 mast cells was reverse-transcribed to cDNA using the SuperScript™ IV First-Strand Synthesis System. qPCR was performed using a LightCycler®96 Instrument (Roche) and the FastStart Essential DNA Green Master. The primer sequences are listed in Table 1. Acquisition on the SYBR green was recorded at the end of the extension step. The melting peaks of target genes were obtained with temperatures ranging from 81 to 90 °C (Fig. S2). All data were acquired using the LightCycler®96 software, and relative mRNA was normalized using the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Fig. S3). The expression levels were analyzed using the $2^{-\Delta\Delta CT}$ method [19].

Protein Extraction and Western Blotting

The total protein in RBL-2H3 mast cells was extracted using RIPA lysis buffer containing phosphatase and protease inhibitors [20]. The extracted proteins were quantified using the Pierce BCA Protein Assay Kit. Western blotting was performed as previously described [21]. Briefly, 10 µg of protein was loaded onto 8% Tris–HCl gels for sodium dodecyl sulfate-poly acrylamide gel electrophoresis. The proteins were transferred to nitrocellulose membrane (Millipore, Middlesex County, MA, USA), washed, and blocked used by 5% skim milk in PBS-Tween buffer (PBST, pH 7.4) for 1 h. After blocking, the membranes were incubated with primary antibodies (STAT6, and pSTAT6, 1:1000) overnight. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (1:10,000 in PBST) for 1 h. Protein expression was analyzed using Amersham™ Imager 600 with the WesternBright ECL kit.

Hemolytic and Bile Salt Hydrolase Activity

Hemolysis of each LAB strains was determined according to the method described in previous report [22]. LAB was cultured in 5% sheep blood on tryptic soy agar to assess the colonies; alpha (green colony), beta (clean zone), or gamma (no change). BSH activity of LAB was measured in MRS agar containing 0.5% (w/v) taurodeoxycholic acid (sodium salt) hydrate and 0.037% $CaCl_2$ at 37 °C, for 48 h [23]. If LAB exhibits BSH activity, a precipitation zone surrounding the colony appears.

Simulated Gastrointestinal Tract

The survival rate of LAB in the simulated gastrointestinal tract was evaluated using a previously reported method [24]. After culturing the LAB for 18 h, they were washed twice with PBS (pH 7.4) and resuspended to 0.95–1.05 at 600 nm. Then, 1 mL of the solution was mixed with 9 mL of PBS (pH 2.5) containing 0.3% pepsin (simulated gastric fluid, SGF) and incubated at 37 °C with gentle shaking at 200 rpm. After 2 h, the pellet was obtained using centrifugation at 4000 rpm for 5 min. Subsequently, 10 mL (pH 7.4) of 1% pancreatin and 1% bile salt (stimulated intestinal fluid, SIF) solutions were added to the pellet and incubated at 37 °C for 2.5 h with gentle shaking at 200 rpm. The number of viable LAB was counted using MRS agar, and the survival rate was calculated as the percentage of viable cells compared to the initial number of cells.

Adhesion to HT-29 Intestinal Epithelial Cells

The adhesion of LAB to HT-29 cells was evaluated as previously described [22]. HT-29 cells (2×10^5 cells/mL) were seeded in 12-well plates and incubated at 37 °C and 5% CO_2

Table 1 Primer sequences of genes used in this study

Gene		Sequence (from 5′ to 3′)	
		Sense	Antisense
RAW 264.7 macrophages (mouse)	*IL-6*	TACCACTTCACAAGTCGGAGGC	CTGCAAGTGCATCATCGTTGTTC
	TNF-α	GGTGCCTATGTCTCAGCCTCTT	GCCATAGAACTGATGAGAGGGAG
	GAPDH	TCTCCCTCACAATTTCCATCC	GGGTGCAGCGAACTTTATTG
	IL-4	TCCTTACGGCAACAAGGAAC	GTGAGTTCAGACCGCTGACA
RBL-2H3 mast cells (rat)	*IL-5*	GATGAGGCTTCCTGTTCCTACT	TGACAAGTTTTGGAATAGTATTTCC
	IL-13	AACAGCAGCATGGTATGGAGCG	TGGGTCCTGTGGATGGCATTGC
	GAPDH	ATGGGAAGCTGGTCATCAAC	GTGGTTCACACCCATCACAA

IL-1β interleukin 1 beta, *IL-6* interleukin 6, *TNF-α* tumor necrosis factor alpha, *IL-4* interleukin 4, *IL-5* interleukin 5, *IL-13* interleukin 13, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase

for 24 h. LAB strains were cultured in MRS broth at 37 °C for 24 h. The LAB strains were resuspended at 1×10^8 CFU/mL in DMEM without FBS and P/S, and treated with HT-29 cells. After 2 h, the HT-29 cells were washed twice with PBS to detach non-adherent LAB. The number of viable LAB was measured by counting on MRS agar and was calculated as log CFU/mL.

Statistical Analysis

Results are expressed as the mean ± standard error of the mean (SEM). Statistical significance was analyzed using the Student's *t*-test, and the Duncan's multiple range tests with the IBM® Statistical Package for the Social Sciences Statistics (ver. 21.0 for Window, IBM, Armonk, NY, USA). Statistical significance was set at $P < 0.05$.

Results

Inhibition of Pro-inflammatory Cytokine Expression by *L. gasseri* and *L. paracasei* SL in LPS-Induced RAW 264.7 Macrophages

In order to establish a concentration that does not show cytotoxicity, MTT assay was performed with 2.5, 5, and 10% of SL treating in RAW 264.7 macrophages. The 2.5 and 5% SL in SFM exhibited no cytotoxicity (≥ 100%) on RAW 264.7 macrophages (Fig. 1a). With these results, SL 5% was chosen for subsequent experiments. To verify that SL affected pro-inflammation signaling, we examined mRNA expressions of *IL-6* and *TNFα*, which lead to hyperimmune responses in LPS-induced RAW 264.7 macrophages (Fig. 1b and c). *IL-6* and *TNF-α* mRNA levels in LPS-induced macrophages were significantly higher than those in untreated cells. *IL-6* and *TNF-α* mRNA expressions were significantly downregulated by 0.33–0.61 fold and 0.35–0.75 fold in LPS-induced RAW 264.7 macrophages treated with SL, respectively.

Reduction of IL-4, IL-5, and IL-13 Cytokines by SL from *L. gasseri* and *L. paracasei* in DNP-IgE Sensitized RBL-2H3 Mast Cells

The 5% SL in SFM showed no cytotoxicity (≥ 93%) in RBL-2H3 cells stimulated with DNP-IgE/HSA (Fig. 2a). As with Raw 264.7 macrophage, SL 5% was chosen for subsequent experiments. To verify whether SL might affect the cytokine expression in mast cells, *IL-4*, *IL-5*, and *IL-13* mRNA expression was examined in DNP-IgE/HSA-sensitized RBL-2H3 mast cells (Fig. 2b–d). Exposure to DNP-IgE/HSA increased *IL-4* (47.10-fold), *IL-5* (1.66-fold), and *IL-13* (4.84-fold) mRNA expression compared with that in non-sensitized RBL-2H3 mast cells; however, treatment with SL significantly decreased

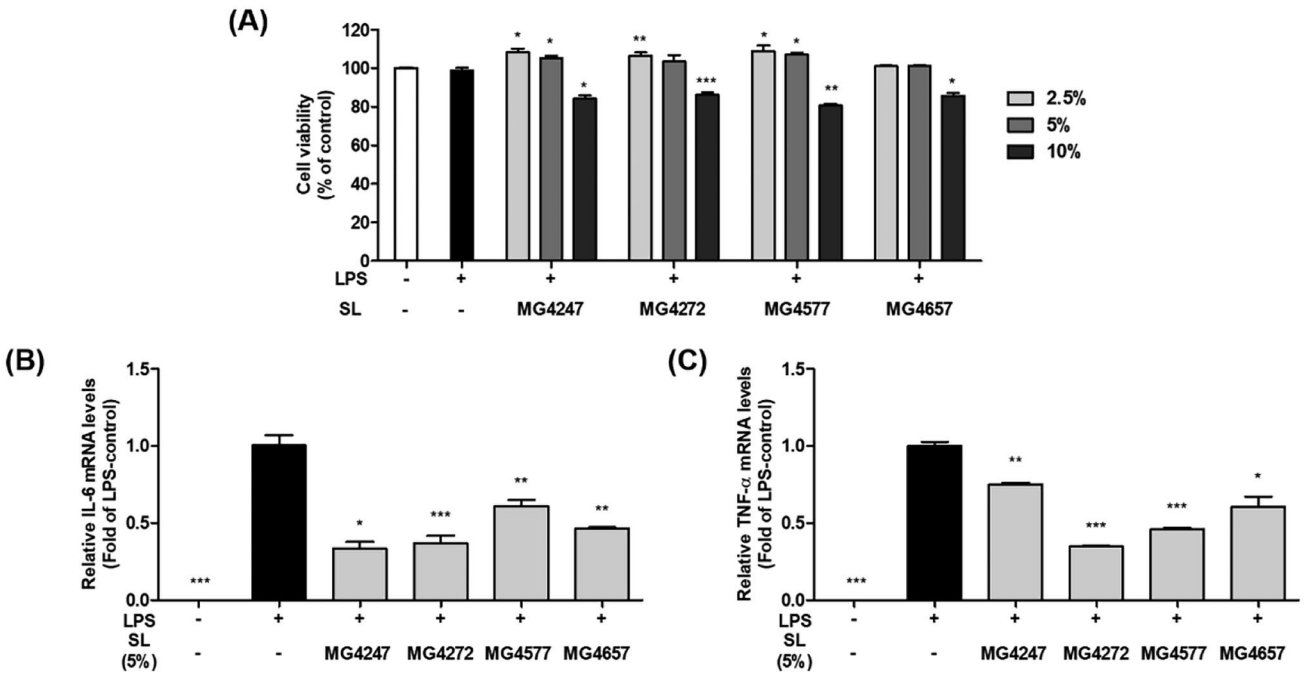

Fig. 1 Cell viability and pro-inflammatory cytokines expression of LPS-induced RAW 264.7 macrophages treated with SL. **A** Cell viability was assessed using the MTT assay in RAW 264.7 macrophages pretreated with SL (2.5, 5, and 10%) for 1 h and then LPS (0.1 μg/mL) with SL treatment for 24 h. Data has no statistical significance. The mRNA expression of pro-inflammatory cytokines, **B** IL-6, and **C** TNF-α, was evaluated using qPCR. Gene expression was normalized with *GAPDH*. RAW 264.7 macrophages were pretreated with LPS (0.1 μg/mL) and treated with SL for 3 h. Data are presented as the means ± SEM ($n = 3$). Statistical Significance was determined by one-way analysis of variance according to Student's *t*-test; $^{**}p < 0.01$, $^{***}p < 0.001$ vs. non-treated control

the mRNA expression of all cytokines (*IL-4*, 0.30–0.81 fold; *IL-5*, 0.46–0.69 fold; and *IL-13*, 0.76–0.91 fold), except for *IL-13*, in MG4657 cells. Therefore, MG4247, MG4272, and MG4577 were selected for the subsequent studies.

Effect on STAT6 Phosphorylation by SL from *L. gasseri* and *L. paracasei* in DNP-IgE Sensitized RBL-2H3 Cells

Western blotting was performed to determine whether the LAB candidates affected STAT6 phosphorylation, which leads to the secretion of IL-4, IL-5, and IL-13 in DNP-IgE/HSA-sensitized RBL-2H3 mast cells (Fig. 3a). Exposure of RBL-2H3 mast cells to DNP-IgE/HSA markedly upregulated the expression of p-STAT6/STAT6 (31.49-folds) compared to that in untreated mast cells. SL treatment of RBL-2H3 mast cells induced with DNP-IgE/HSA reduced the expression of p-STAT6/STAT6 (18.36 to 25.91-fold) (Fig. 3b).

Safety of *L. gasseri* MG4247, *L. paracasei* MG4272, and MG4577 as probiotics

To investigate the safety of MG4247, MG4272, and MG4577, which alleviated the effects of cytokines associated with allergic symptoms, hemolysis, BSH, and cytotoxicity on intestinal epithelial cells were measured. MG4247, MG4272, and MG4577 did not show β-hemolysis (data not shown), indicating no hemolytic activity on the host. In terms of BSH activity, MG4247, MG4272, and MG4577 showed no precipitation (Fig. 4a). Moreover, MG4247, MG4272, and MG4577 (1×10^6–10^8 cells/mL) were not significantly toxic, indicating that none of the LAB damaged the intestinal epithelium after adherence (Fig. 4b).

Stability of *L. gasseri* MG4247, *L. paracasei* MG4272, and MG4577 in Stimulated GIT

The viability of *L. gasseri* MG4247 *L. paracasei* MG4272, and MG4577, which exhibited an anti-allergy effect in this study, was determined using artificial GIT (Table 2). Initial *L. gasseri* MG4247, *L. paracasei* MG4272, and MG4577 numbers at OD_{600} were determined as 8.03, 8.92, and 9.66 Log CFU/mL. In stimulated SGF (at pH 2.5 with 0.3% pepsin), all strains survived ≥ 75%. In stimulated SIF with surviving strains in SGF, *L. gasseri* MG4247, *L. paracasei* MG4272, and MG4577 showed 96.18, 87.18, and 73.90% viability.

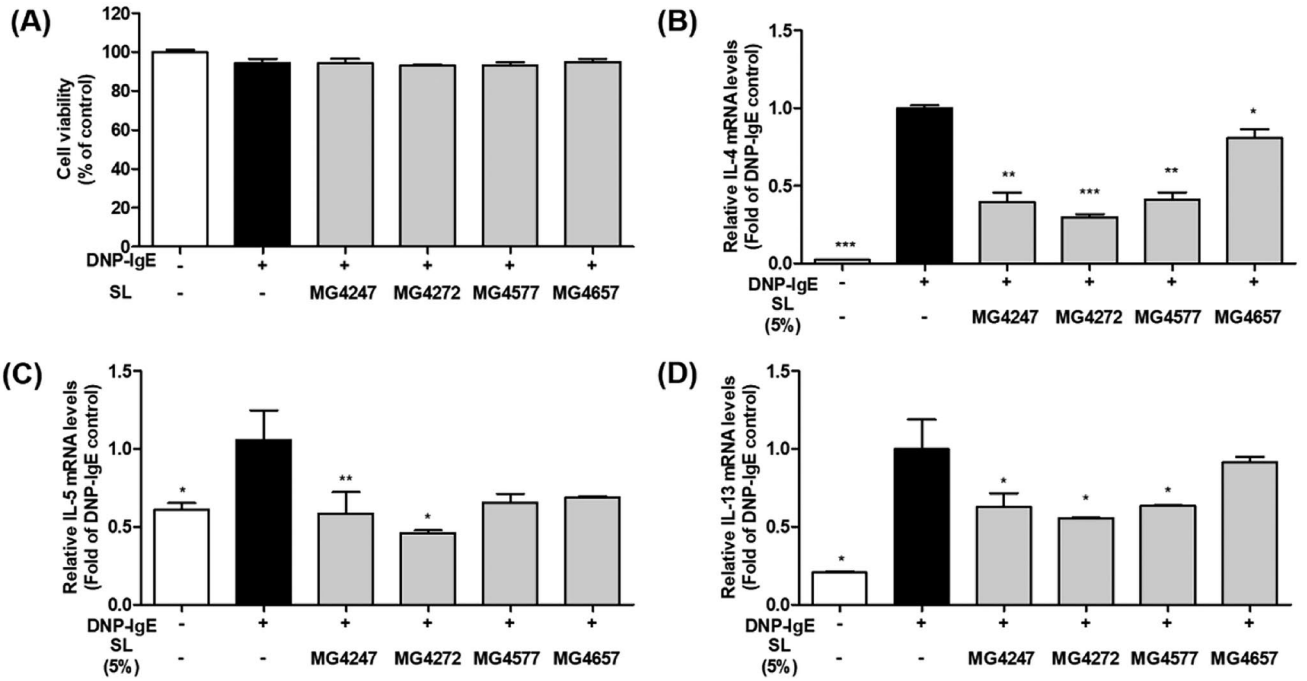

Fig. 2 Effect of SL on viability and cytokines levels in DNP-IgE/HSA-sensitized RBL-2H3 mast cells. **A** Cell viability was assessed using the MTT assay in RBL-2H3 cells treated with anti-DNP-IgE (110 ng/ml) for 1 h, followed by stimulation with SL (5%) for 24 h. The mRNA expression of cytokines, **B** *IL-4*, **C** *IL-5*, and **D** *IL-13*, was evaluated using qPCR in DNP-IgE/HSA induced RBL-2H3 mast cell. Each gene expression was measured in RBL-2H3 cells treated with anti-DNP-IgE (110 ng/ml) for 1 h, followed by stimulation with SL (5%) for *IL-4* and *IL-13* for 1 h, and *IL-5* (3 h), and then with 25 ng/mL DNP-HSA antigen (25 ng/ml). Gene expression was normalized with *GAPDH*. Data are presented as the mean ± SEM ($n = 3$). Statistical significance was determined by one-way analysis of variance according to Student's *t*-test; $^{**}p < 0.01$, $^{***}p < 0.001$ vs. non-treated control

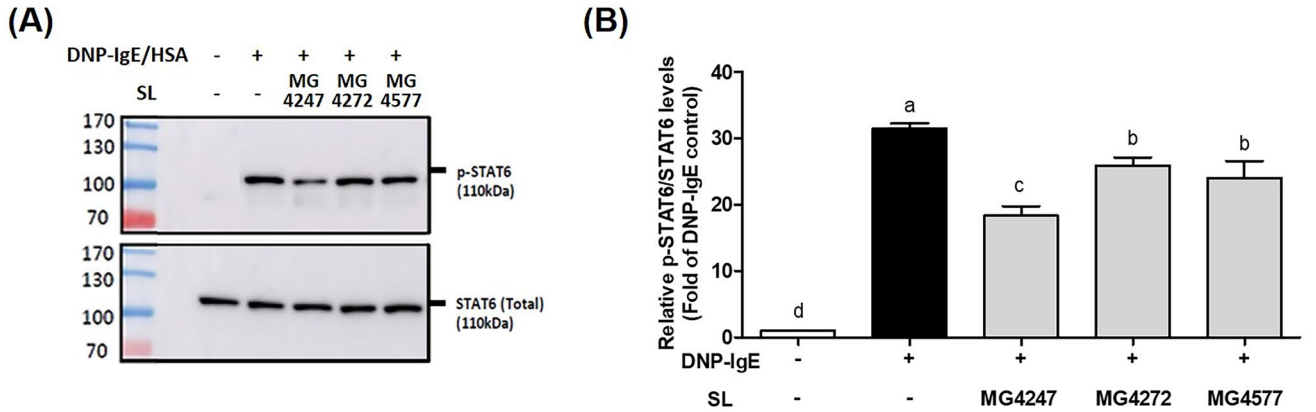

Fig. 3 Effects of SL on STAT6 phosphorylation in DNP-IgE/HSA-sensitized RBL-2H3 mast cell. RBL-2H3 cells were treated with anti-DNP-IgE (110 ng/ml) for 1 h, followed by stimulation with SL (5%) for 1 h, and then with DNP-HSA antigen (25 ng/ml). **A** STAT6 and p-STAT6 levels were detected by western blotting. **B** The graphs show STAT6 protein expression relative to no phosphorylation. Data are presented as the mean ± SEM ($n = 3$). Different letters on each column indicate statistical significance

Adhesion of L. gasseri MG4247, L. paracasei MG4272, and MG4577 to HT-29 Intestinal Epithelial Cells

To determine the ability to adhere to intestinal epithelial cells, LAB attachment to HT-29 cells was determined using plate counting (Fig. 5). As a result of expressing the attached *L. gasseri* MG4247, *L. paracasei* MG4272, and MG4577 as a percentage in proportion to the initial counts, all strain were confirmed to be 66% or more adherent to HT-29 intestinal epithelial cells.

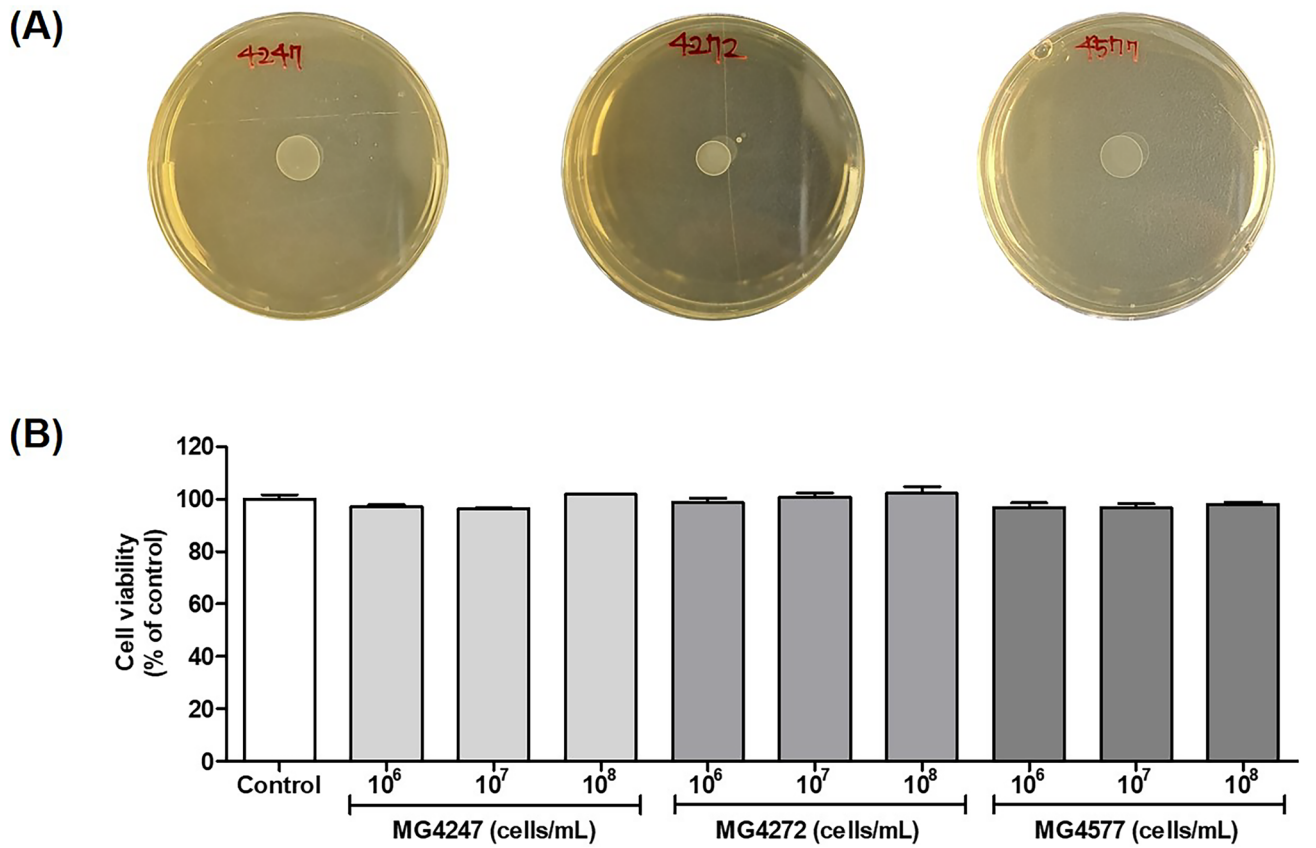

Fig. 4 Safety tests of LAB as probiotics. **A** BSH activity of *L. gasseri* MG4247 and *L. paracasei* MG4272 and MG4577. **B** Cell viability of HT-29 intestinal epithelial cells treated with *L. gasseri* MG4247 and *L. paracasei* MG4272 and MG4577 (1×10^6, 10^7, and 10^8 cells/mL). Data are presented as the mean ± SEM ($n = 3$). Data has no statistical significance

Table 2 Tolerance of stimulated GIT to *L. gasseri* MG4247, *L. paracasei* MG4272, and MG4577

Strains	Initial counts (Log CFU/mL)	SGF (Log CFU/mL)	SIF (Log CFU/mL)	Survival rate (%)
L. gasseri MG4247	8.03 ± 0.01	7.85 ± 0.02	7.73 ± 0.04	96.18 ± 0.51
L. paracasei MG4272	8.92 ± 0.01	7.96 ± 0.01	7.77 ± 0.01	87.18 ± 0.11
L. paracasei MG4577	9.66 ± 0.03	7.25 ± 0.03	7.14 ± 0.01	73.90 ± 0.50

Simulated gastric fluid (SGF) contained 0.3% pepsin at pH 2.5, *stimulated intestinal fluid (SIF)* contained 1% pancreatin and 1% bile salt at pH 7.4

Data are presented as the mean ± standard deviation (duplicate)

Discussion

Allergic inflammatory responses are stimulated by various environmental factors, including pollen, food, and climatic conditions If allergic responses are untreated, they can lead to bronchitis, pneumonia, asthma, or chronic obstructive pulmonary disease (COPD) [25]. Hence, allergic inflammation should be treated before it progresses to COPD or other diseases. Probiotics not only strengthen the host's gut immunity but also attenuate inflammatory and allergic diseases [11]. In patients with allergic diseases, the gut microbiota is altered; in comparison with healthy individuals, in patients with allergic diseases, the relative abundance of *Lactobacillus* and *Bifidobacterium* is reduced, whereas the abundance of pathogens such as *Escherichia coli*, *Clostridium difficile*, and *Staphylococcus aureus* is increased [26]. In particular, metabolites such as short-chain fatty acids produced by probiotics regulate allergic inflammation through the G protein-coupled receptors in intestinal epithelial cells [27]. Therefore, consuming probiotics with potential as therapeutic agents for allergy symptoms can be a more effective alternative to using the native gut microbiota. When LAB is cultured in MRS, which is the most used to confirm the metabolite difference of LAB, metabolite giving beneficial effect to human is present in the supernatant of MRS medium [28]. It has been reported that various metabolites such as SCFA are produced when probiotics are taken and stabilized in the intestine in human [29]. In addition, different metabolites, even belonging to the same species, are produced in each strain [28]. This study assessed the anti-allergy effects of SL from LAB on pro-inflammatory cytokine levels in macrophages and mast cells. Moreover, we selected LAB as functional probiotic candidates and evaluated their stability and safety as probiotics.

In allergic inflammation, the major cytokines that stimulate inflammation are Th1 and Th2 cytokines [4]. Th1 cytokines, especially TNF-α and IL-6, induce inflammation in macrophages [5]. LPS, which binds to the toll-like receptor 4 in the cell membrane, promotes the nuclear factor kappa-light-chain-enhancer of activated B cells signaling pathway. Because of this pathway, macrophages produce TNF-α- and IL-6 cytokines and Th1 cells undergo differentiated [30]. Our results showed that all LAB strains significantly decreased the mRNA expression of *IL-6* and *TNF-α*. Th2 cytokines are known to cause allergic inflammation by increasing the production of immunoglobulin E (IgE) in B cells [31]. In this study, MG4247, MG4272, and MG4577 reduced the mRNA levels of Th2 cytokines, including *IL-4*, *IL-5*, and *IL-13*. Similarly, many *Lactobacillus* strains have shown anti-allergy effects by modulating Th2 cytokines in cells and animal model [32]. IL-4/13 cytokines, which are attached to the IL-4/13 receptor, induce STAT6 protein expression in mast cells [33]. JAKs, activated by IL-4/13 cytokines, phosphorylate STAT6, which abnormally secretes

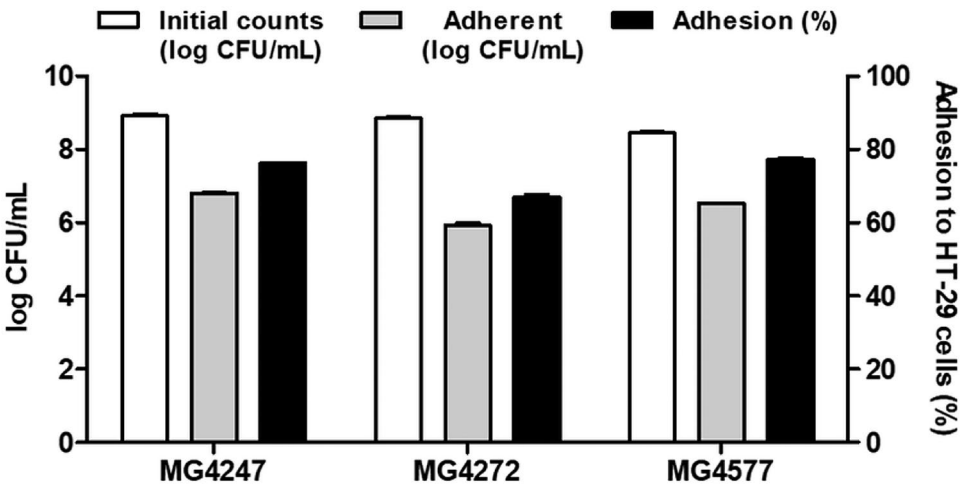

Fig. 5 Adhesion of *L. gasseri* MG4247, *L. paracasei* MG4272, and MG4577 to HT-29 intestinal epithelial cells. Data are presented as the mean ± SEM ($n = 3$)

Th2 cytokines and initiates allergic inflammation [34]. The p-STAT6, which produces Th2 cytokines, was decreased by MG4247, MG4272, and MG4577 treatment. Comparing the difference between both LAB strains, it was confirmed that both strains significantly lowered Th1 cytokines; however, in Th2 cytokines, especially *L. paracasei* was significantly reduced. Considering our results, MG4247, MG4272, and MG4577 showed anti-allergy effects by regulating Th1 and Th2 cytokine levels in macrophages and mast cells.

The in vitro properties of certified probiotics include BSH activity, hemolysis, resistance to gastric acidity, and basic conditions with bile acid, and adherence to epithelial cells [35]. Therefore, stability and safety as probiotics of MG4247, MG4272, and MG4577 with anti-allergic effect were investigated. Probiotics have been reported to have various beneficial effects on the host when they reach the intestines after ingestion [36]. Therefore, it is necessary for the probiotics to have the ability to survive in the GIT. In this study, MG4247, MG4272, and MG4577 showed survival rates more than 73.90% in simulated GIT. Other commercial probiotics, including *Lactobacillus* and *Bifidobacterium* sp. exhibited 51.72–93.90% survival rate after passing the simulated GIT [36]. In particular, *L. paracasei* was reported to exhibit 37.60 to 79.88% viability in simulated GIT [37]. Our results demonstrate similar viability of LAB in artificial GIT compared to other probiotics. In addition, probiotics attached to the intestinal mucosa promote various beneficial effects by producing metabolites and stimulating the gut barrier in the host [38]. First, we established that MG4247, MG4272, and MG4577 were non-toxic and adherent to intestinal epithelial cells. The adhesion of MG4247, MG4272, and MG4577 to intestinal epithelial cells was more than 66%. Other probiotics, which were distributed recently, were reported to be 60.2% adherent to colon cells [39]. Compared to the results of cited literature, it is suggested that MG4247, MG4272, and MG4577 may have a higher adhesion rate in intestinal epithelial cells. Although it has been scientifically verified in vitro that *L. gasseri* MG4272, *L. paracasei* MG4247, and MG4577 down-expressed allergy-related cytokines, further studies in animal models sensitized by various allergens or/and clinical trials still need to be performed.

Conclusions

Our results suggest that LAB are effective in modulating allergic inflammation in macrophages and mast cells. In the present study, treatment with LAB reduced TNF-α and IL-6 expression levels in LPS-induced RAW 264.7 macrophages and the levels of Th2 cytokines, IL-4, IL-5, and IL-13, by reducing p-STAT6 protein expression in DNP-IgE/HSA-sensitized RBL-2H3 mast cells (Fig. 6). Among LAB, *L. gasseri* MG4272 and *L. paracasei* MG4247 and

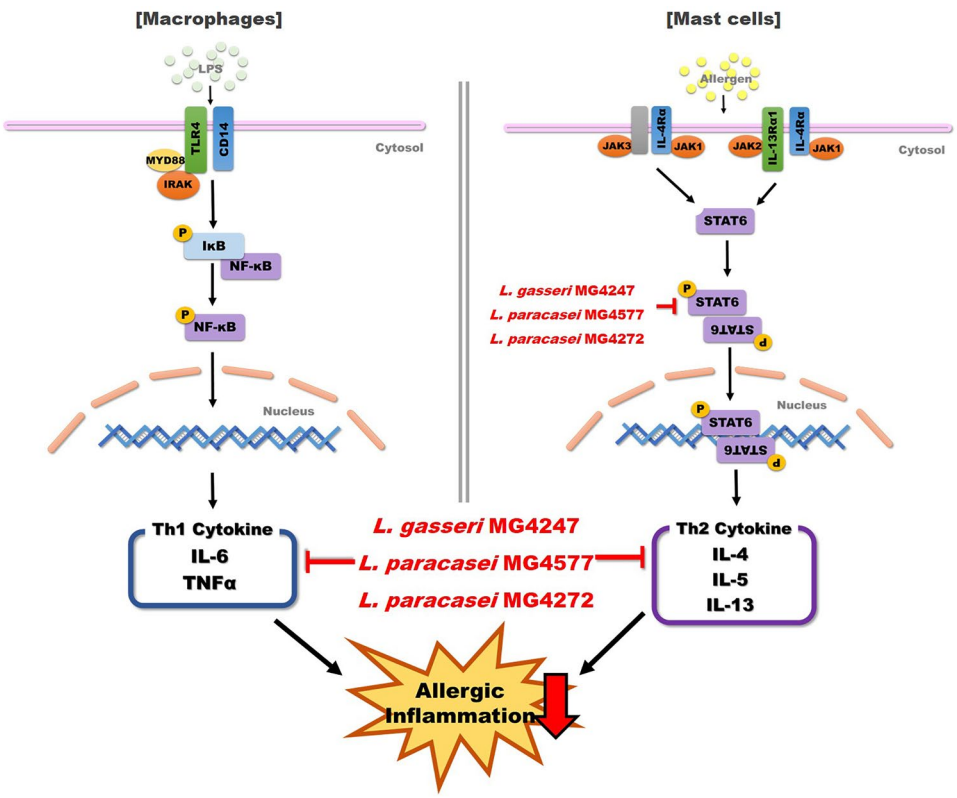

Fig. 6 *L. gasseri* MG4247 and *L. paracasei* MG4577 reduced allergic symptoms via modulating phosphorylation of STAT6, Th1, and Th2 cytokines in macrophages, and mast cells

MG4577 can be qualified as probiotics because of their safety and stability in the gastric intestine of the host. Thus, *L. gasseri* MG4272, *L. paracasei* MG4247, and MG4577 secured have safety as a probiotic, and these strains decrease the Th1 cytokines production, and also suppress secretion of Th2 cytokines via reduction of pSTAT6 phosphorylation.

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Data Availability The authors declare that all data and materials support published claims and comply with field standards.

Declarations

Ethics Approval This article does not contain any studies with human or animal subjects.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflict of Interest J.Y. Lee and C.-H. Kang are currently employed by the MEDIOGEN Corporation, Republic of Korea. None of the other authors had any conflict of interests.

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