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ARTICLE

Efficacies of Potential Probiotic Candidates Isolated from Traditional Fermented Korean Foods in Stimulating Immunoglobulin A Secretion

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Abstract   The aim of this study was to evaluate efficacies of selected lactic acid bacteria (LAB) in inducing immunoglobulin A (IgA) secretion. Twenty-five different LAB isolated from traditional fermented Korean foods were characterized for their probiotic properties and screened to identify those that could stimulate lamina propria cells (LPCs) from Peyer's patch to secret IgA *in vitro*. Among them, four strains (*Lactiplantibacillus plantarum* CJW55-10, *Lactiplantibacillus pentosus* CJW18-6, *L. pentosus* CJW56-11, and *Pediococcus acidilactici* CJN2696) were found to be strong IgA inducers. The number of IgA positive B cells and soluble IgA level were increased when LPCs were co-cultured with these LAB. Expression levels of toll-like receptor (TLR) such as TLR2 and TLR4 and secretion of interleuckin-6 were augmented in LPCs treated with these LAB. Further, we determined whether oral intake of these LAB enhanced IgA production *in vivo*. After one-week of daily oral administration, these LAB feed mice increased mucosal IgA and serum IgA. In conclusion, selected strains of LAB could induce systemic IgA secretion by activating lamina propria B cells in Peyer's patch and oral intake of selected strains of LAB can enhance systemic immunity by inducing mucosal IgA secretion.

Keywords   interleukin-6, immunoglobulin A, lactic acid bacteria, Peyer's patch, toll-like receptor

Introduction

The mucosal immunity serves as the first defense line against pathogenic organisms that can penetrate the physical barrier (Takahashi et al., 2021). Gut-associated lymphoid tissue (GALT), a specialized secondary lymphoid tissue around the intestine, is often referred as a model tissue to visualize the critical function of mucosal immunity because the interaction between gut bacteria and immune cells in GALT can

affect both local mucosal immunity and systemic immunity (Mörbe et al., 2021).

Immunoglobulin A (IgA) is produced when immune cells within GALT are activated by foreign antigens (Mörbe et al., 2021). It has been proposed that initial production of IgA is caused by exposure to commensal bacteria soon after birth (Chen et al., 2020). IgA provides many beneficial aspects in the mucosal immunity against foreign antigens mainly by exhibiting neutralizing activities against gut bacteria or their soluble factors (Chen et al., 2020). Supporting this idea, IgA deficiency in humans is associated with various diseases including infection, autoimmune disease, allergy, and cancer (Swain et al., 2019).

Recent evidence suggests that administration of certain strain of lactic acid bacteria (LAB) such as *Lacticaseibacillus rhamnosus* GG can facilitate IgA secretion by stimulating immune cells within GALT (Jin et al., 2021). Moreover, IgA induction by *Lactiplantibacillus pentosus* b240 can make mice become more resistant to influenza virus or *Streptococcus pneumoniae* infection (Kiso et al., 2013; Tanaka et al., 2011). These results implied that produced IgA after stimulation with LAB in GALT could protect infection against microbial pathogens through systemic circulation. Therefore, the oral intake of certain LAB might benefit the host through increased protection against certain microbial pathogens by enhancing mucosal immunity. However, most of study describing the enhancement of IgA production by LAB just focused on the efficacy of LAB without any mechanical insights.

In this study, we screened 25 different strains of LAB isolated from traditional fermented Korean foods based on their abilities to induce IgA secretion *in vitro* and *in vivo*. Results clearly indicated that some of LAB stimulate lamina propria cells (LPCs) from Peyer's patch to secret IgA via toll-like receptor (TLR) / interleuckin-6 (IL-6) axis. Further, we demonstrated that oral stimulation by these LAB increased systemic IgA levels including levels of GALT IgA, bronchus-associated lymphoid tissue (BALT) IgA and serum IgA in mice.

Materials and Methods

Isolation, identification and characterization of lactic acid bacteria (LAB)

Twenty-five different strains of LAB were isolated from several traditional fermented Korean foods according to previously established method with a slight modification (Lee et al., 2011). Briefly, modified MRS agar plates (pH 5.0; BD Biosciences, San Diego, CA, USA) were used for culturing all samples at 37℃ for 48 h under anaerobic conditions to enrich LAB. Single colonies were selected from these plates, cultured again on MRS broth (BD Biosciences), and characterized by Gram-staining and utilization of carbohydrate source. For species identification, 16S rDNA of each strain of LAB was sequenced and aligned using an established DNA database (http://www.ncbi.nlm.nih.gov/BLAST; Lee et al., 2011). Identified species and strain names are listed in Table 1. Each strain of LAB was sub-cultured more than three times before experiments.

pH and bile tolerance

Growths of each strain of LAB under low pH and bile content were measured according to previously established method (Lee et al., 2011). Briefly, each strain of LAB (1.0×10^8 CFU/mL) was cultured in acidic MRS broth (final pH 2.5) containing 1,000 units/mL of pepsin (Sigma-Aldrich, St. Louis, MO, USA) for 3 h at 37℃ or MRS broth containing 0.3% oxgall (Sigma-Aldrich) for 24 h at 37℃. After culture, 100 μL of broth was plated in triplicate onto MRS agar to measure final CFU.

Table 1. Characterization of strains of LAB isolated from traditional fermented Korean foods

| Strain | Species[1] | Growth/survival[2] | | Adhesion (%)[3] | Hemolytic pattern | Urease activity | Gelatinase activity | IgA secretion (ng/mL)[4][5] | Source |
		pH 2.5	Oxgall 0.3%						
CJT1088	*Lactiplantibacillus plantarum*	Negative	Negative	83	Gamma	Negative	Negative	34±20.8	Doenjang (fermented soybean paste)
NR02	*L. plantarum*	Negative	Negative	84	Gamma	Negative	Negative	93±24.2	Kimchi
CJIH53	*L. plantarum*	Negative	Positive	61	Gamma	Negative	Negative	99±28.7	Kimchi
CJT1085	*L. plantarum*	Negative	Positive	87	Gamma	Negative	Negative	34±18.3	Kimchi
KKK412	*Limosilactobacillus fermentum*	Negative	Positive	77	Gamma	Negative	Negative	35±18.8	Kimchi
KJP421	*L. plantarum*	Negative	Positive	84	Gamma	Negative	Negative	35±1.0	Kimchi
CJT1172	*Lacticaseibacillus casei*	Negative	Positive	76	Gamma	Negative	Negative	53±3.6	Kimchi
CJT1305	*L. casei*	Negative	Positive	77	Gamma	Negative	Negative	47±3.5	Kimchi
CJN1917	*L. plantarum*	Negative	Negative	64	Gamma	Negative	Negative	61±9.5	Maggeolli (rice wine)
CJN2694	*L. casei*	Negative	Positive	68	Gamma	Negative	Negative	58±0.9	Maggeolli (rice wine)
CJN2696	*Pediococcus acidilactici*	Negative	Negative	69	Gamma	Negative	Negative	139±2.2	Maggeolli (rice wine)
CJW18-6	*Lactiplantibacillus pentosus*	Negative	Positive	70	Gamma	Negative	Negative	125±29.6	Fermented green tea
CJW55-10	*L. plantarum*	Negative	Positive	82	Gamma	Negative	Negative	143±54.1	Kimchi
CJW15-5	*L. pentosus*	Negative	Positive	60	Gamma	Negative	Negative	107±7.3	Kimchi
CJW18-3	*L. plantarum*	Negative	Positive	68	Gamma	Negative	Negative	101±5.6	Fermented green tea
CJW26-3	*L. pentosus*	Negative	Positive	70	Gamma	Negative	Negative	99±11.3	Kimchi
CJW41-7	*L. plantarum*	Negative	Positive	64	Gamma	Negative	Negative	106±2.8	Kimchi
CJW54-15	*Companilactobacillus farciminis*	Negative	Positive	ND	Gamma	Negative	Negative	86±1.4	Kimchi
CJW55-1	*L. pentosus*	Negative	Positive	69	Gamma	Negative	Negative	105±7.3	Kimchi
CJW56-3	*L. pentosus*	Negative	Positive	90	Gamma	Negative	Negative	100±13.0	Kimchi
CJN2359	*L. plantarum*	Negative	Negative	64	Gamma	Negative	Negative	94±13.0	Maggeolli (rice wine)
CJW20-4	*Levilactobacillus brevis*	Negative	Positive	49	Gamma	Negative	Negative	97±2.8	Kimchi
CJW41-5	*L. plantarum*	Negative	Positive	88	Gamma	Negative	Negative	87±1.1	Kimchi
CJW55-8	*L. plantarum*	Negative	Positive	68	Gamma	Negative	Negative	99±3.9	Kimchi
CJW56-11	*L. pentosus*	Negative	Positive	70	Gamma	Negative	Negative	124±7.3	Kimchi

[1] 16s rDNA sequenced having over 99% homology.
[2] Positive, growth or survival; negative, no growth or survival compared with initial inoculums. Initial inoculums at approximately 1×10^8 CFU/mL.
[3] Percentage of adhering lactobacilli in HT-29 cell after 3 h incubation. Initial inoculums at approximately 1×10^8 CFU/mL.
[4] Results were shown as means±SEs.
[5] IgA secretion without LAB stimulation was 22±3.0 ng/mL.
LAB, lactic acid bacteria; IgA, immunoglobulin A; ND, not determined.

Adhesion assay

For adhesion assay, 4×10^4 cells/well of HT-29 cells (human epithelial cells; American Type Culture Collection, Manassas, VA, USA) in RPMI-1640 (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich) were plated into 24-well tissue culture plates. These cells were then incubated with 1×10^8 CFU/mL of each LAB strain at 37°C. After 3 h, cells were washed with PBS six times. After washing, bacteria that attached to HT-29 cells were harvested by repeatedly pipetting with chilled sterile water and cultured on MRS agar plates to measure final CFU. Sources, probiotic properties of identified

species and strain names are summarized in Table 1. Each strain of LAB was sub-cultured more than three times prior to *in vitro* experimental analysis.

Safety assessment of lactic acid bacteria (LAB)

For measuring hemolytic activity, each strain of LAB was inoculated on BD BBL™ prepared plated medium (Trypticase™ soy agar with 5% sheep blood; Thermo Fisher Scientific, Waltham, MA, USA) and incubated at 37℃ for 48 h. After 48 h, the colonies on plates were characterized to observe the hemolytic pattern (Fu et al., 2022). To detect urease activity, each strain of LAB was inoculated on Remel™ urea agar base (Thermo Fisher Scientific) and incubated at 37℃ for 48 h. After 48 h, the color changes of plates were monitored to observe the urease activity (Christensen, 1946). For gelatin liquefaction test, each strain of LAB ($5.0×10^6$ CFU/mL) was cultured in MRS gelatin broth containing 12% (w/v) of gelatin for 48 h at 37℃. After 48 h, the cultured MRS gelatin broths were incubated at 4℃ to monitor gelatin liquefaction (Fugaban et al., 2022).

Animals

BALB/c, C3H/HeJ, and C3H/HeN mice (female, 6–8 weeks old) were purchased from Central Lab Animal Incorporation (Seoul, Korea) and acclimated for one week prior to experiments. Mice were fed a standard rodent diet with purified water *ad libitum* and kept at 20℃–24℃ with 40%–60% humidity in Korea University animal facility under a 12 h/12 h light-dark cycle. Mice received proper care in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Korea University (protocol numbers: KUIACUC-2016-160 and KUIACUC-2017-107).

Analysis of lactic acid bacteria (LAB)-stimulated lamina propria cells (LPCs) *in vitro*

LPCs from Peyer's patches were isolated from the small intestine of BALB/c, C3H/HeJ, or C3H/HeN mouse according to previously established method (Kikuchi et al., 2014). Isolated LPCs ($2.5×10^5$ cells/well) were then mixed with each heat-killed LAB ($5×10^6$ CFU/mL) and seeded into 96 well plates in 200 μL of RPMI 1640 medium supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin. After 7 days of incubation, LAB-stimulated LPCs were analyzed for TLR2 and TLR4 expression by quantitative real-time polymerase chain reaction (PCR) and visualization of IgA positive B cells (IgA^+B220^+ cells) by flow cytometry analysis. Culture supernatant of each sample after co-culture for 7 days was harvested. Sandwich enzyme-linked immunosorbent assays (ELISAs) were performed to detect IgA (Cat # 88-50450-22, Thermo Fisher Scientific), IL-6 (Cat # BMS603-2, Thermo Fisher Scientific), poly Ig receptor (pIgR; Cat # SEK50119, Sino Biological, Wayne, PA, USA), and transforming growth factor-β (TGF-β; Cat # BMS608-4, Thermo Fisher Scientific) using ELISA kits according to each manufacturer's instructions. For blocking TLR2 signaling, neutralizing antibody against mouse TLR2 (Cat # 121802, BioLegend, San Diego, CA, USA) was added into the LPC culture.

Quantitative real-time polymerase chain reaction (PCR)

Total RNAs were isolated from cultured LPCs and then cDNAs were synthesized using total RNAs, oligo-dT primers, and a First-Strand cDNA Synthesis Kit (SuperScript RT; Thermo Fisher Scientific). Quantitative real-time PCR was then performed with each cDNA as a template using a QGreen™ 2×qPCR Master Mix (GenDEPOT, Katy, TX, USA) on a Bio-Rad CFX96 Real-Time Detection System (Bio-Rad Laboratories, Hercules, CA, USA). PCR primer sequences for mouse *Tlr2* (GenBank accession NM_011905) were as follows: 5'-TTGCTCCTGCGAACTCCTATCC-3' (sense), 5'-AGTCACAC

AGGTAGCTGTCTGG-3' (anti-sense), resulting in a RT-PCR product of 89 bp in length. PCR primer sequences of mouse *Tlr4* (GenBank accession JX878359.1) were as follows: 5'-GCCGGAAGGTTATTGTGGTAGTG-3' (sense), 5'-GGACAAT GA AGATGATGCCAGAGC-3' (anti-sense), resulting in a 123 bp RT-PCR product. Relative expression levels of analyzed genes were normalized to mouse *Gapdh* level. PCR primer sequences of mouse *Gapdh* (GenBank accession NM_008084) were as follows: 5'-ATGGTGAAGGTCGGTGTGAA-3' (sense), 5'-GGTCGTTGATGGCAACAATCTC-3' (anti-sense), resulting in a 100 bp RT-PCR product.

Flow cytometry analysis

To visualize IgA positive B cells (IgA^+B220^+ cells), a single cell suspension from cultured LPCs was stained with FITC-conjugated anti-IgA (Cat # 559354, BD Biosciences, San Jose, CA, USA) and PE-conjugated anti-B220 (Cat # 553090, BD Biosciences). After staining, cells were washed, resuspended in PBS, and analyzed by flow cytometry using a FACSCalibur with CellQuest software (BD Biosciences; Choi et al., 2017).

Oral administration of lactic acid bacteria (LAB) and analyses on animal model

Sixty Balb/c mice were randomly separated into ten groups (n=6 mice per group): Two control (PBS) groups and eight test (LAB) groups. For the two control groups, each group of mice was orally administered 400 µL of PBS every day for 7 days or 21 days, respectively. For the eight test groups, each group of mice was orally administered 400 µL of PBS containing four different strains of LAB (CJW55-10, CJW18-6, CJW56-11, or CJN2696) every day for 7 days or 21 days, respectively. At 7 or 21 days after oral administration, mice were sacrificed to analyze levels of BALT IgA, GALT IgA, serum IgA, and serum IgG using sandwich ELISA kits (Cat # 88-50400-22, Thermo Fisher Scientific). For preparing samples of LAB in animal studies, each strain of cultured LAB was freeze-dried and keep at –80°C. Each sample of LAB ($1×10^{10}$ CFU) was then dissolved in 400 µL of PBS just before oral administration.

Analyses of animal model

To measure serum IgA and IgG levels in experimental animals, blood specimens from experimental mice were collected from infraorbital veins using capillary tubes on day 7 or 21 after the first oral administration of each strain of LAB. To obtain serum samples, each blood sample was incubated on ice for 1 h and centrifuged at 8,000×g for 10 min. Each serum sample was then diluted 1:100 with PBS for measuring IgA and IgG levels (Choi et al., 2017). Sandwich ELISA kits were used to measure total serum IgA (Cat # 88-50450-22, Thermo Fisher Scientific) and IgG (Cat # 88-50400-22, Thermo Fisher Scientific) levels according to the manufacturer's instructions.

To measure BALT IgA, bronchoalveolar lavage was performed with 2 mL PBS after exposing the tracheae of sacrificed mice. Bronchoalveolar lavage fluids were then centrifuged at 800×g for 5 min at 4°C (Choi et al., 2018). Levels of IgA in bronchoalveolar lavage fluids were quantitated using sandwich ELISA kits (Cat # 88-50450-22, Thermo Fisher Scientific).

To measure GALT IgA, LPCs from Peyer's patches were isolated from the small intestine of each sacrificed mouse according to previously established method (Kikuchi et al., 2014). Isolated LPCs ($2.5×10^5$ cells/well) were then mixed with each heat-killed LAB ($5×10^6$ CFU/mL) and seeded into 96-well plates in 200 µL of RPMI 1640 medium supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin. After 2 days of incubation, culture supernatants of each sample were harvested and sandwich ELISAs were performed to detect IgA levels using ELISA kit (Cat # 88-50450-22, Thermo Fisher Scientific).

Statistical analysis

Statistical significance of the experimental data was determined by Student's t-test. Significant differences at a confidence level of 95% and 99% are labeled with asterisks (*) and (**), respectively, on each graph.

Results

Effects of lactic acid bacteria (LAB) isolated from traditional fermented Korean foods on immunoglobulin A (IgA) secretions by lamina propria cells (LPCs) *in vitro*

Twenty-five different strains of LAB were isolated from traditional fermented Korean foods and identified at species level by 16S rDNA sequencing (Table 1). Subsequently, these LAB were characterized for their basic properties as probiotics such as pH tolerance, bile tolerance, and adherence to intestinal epithelial cells (HT-29 cells). All LAB failed to survive or proliferate after three-hour incubation at pH 2.5 (Table 1). However, bile tolerance over 24 h was observed for several strains (Table 1). Also, several strains exhibited adhesive properties to human intestinal epithelial cells (HT-29 cells) after three-hour co-culture (Table 1). Next, hemolytic pattern, urease activity and gelatinase activity of these LAB were determined for the preliminary safety evaluation. All the tested strains exhibited γ-hemolytic pattern without activities of both urease and gelatinase (Table 1). To examine immunomodulatory activity, these LAB were examined for their abilities to stimulate LPCs from Peyer's patches to produce IgA. Among them, *Lactiplantibacillus plantarum* CJW55-10, *L. pentosus* CJW18-6, *L. pentosus* CJW56-11, and *Pediococcus acidilactici* CJN2696 were found to be strong IgA inducers because soluble IgA levels were significantly increased when LPCs were co-cultured with these LAB (Table 1). Supporting this result, IgA secreting B cells (IgA$^+$B220$^+$ cells) were significantly increased when these LAB were co-cultured with LPCs (Fig. 1A). However, total B cells (B220$^+$ cells) of LPCs were not significantly changed after co-culture with these LAB (Fig. 1B).
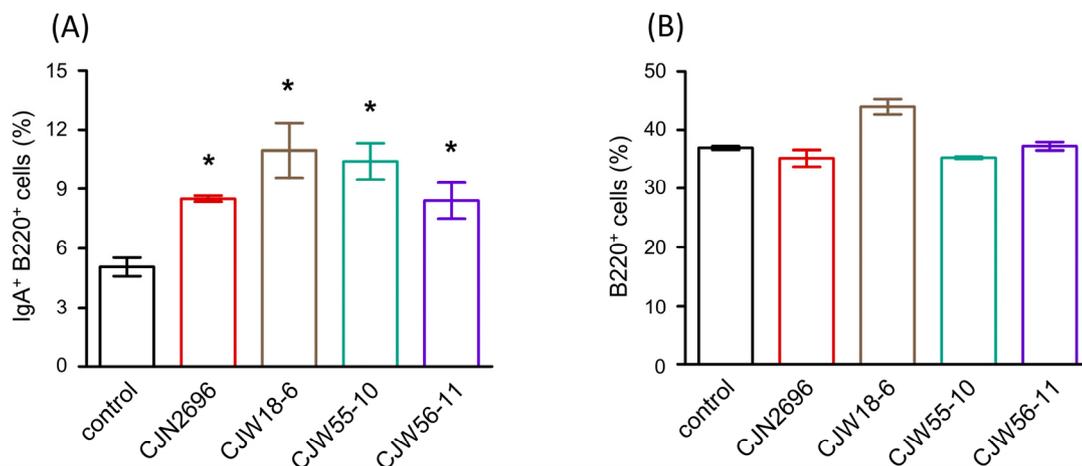
Fig. 1. Selected strains of LAB isolated from traditional fermented Korean foods can increase the frequency of IgA positive B cells within LPCs. LPCs ($2.5×10^5$ cells/well) from Peyer's patches of Balb/c mice were co-cultured with each strain of heat-killed bacterium ($5×10^6$ CFU/mL). After 7 days, flow cytometry analyses were performed with LPCs to detect (A) IgA positive B cells (IgA$^+$B220$^+$ cells) and (B) total B cells (B220$^+$ cells) within LPCs. Control, no treatment. Data are shown as mean±SEM from three independent experiments. Significant differences compared with the control group are indicated by * p<0.05. IgA, immunoglobulin A; LAB, lactic acid bacteria; LPCs, lamina propria cells.

Selected lactic acid bacteria (LAB) can stimulate immunoglobulin A (IgA) and interleuckin-6 (IL-6) secretion through toll-like receptor 2 (TLR2) signaling

To determine how these LAB could stimulate IgA secretion by LPCs, relative expression levels of TLR2 and TLR4 of LPCs stimulated with these LAB were monitored by quantitative real-time PCR analyses because previous observation indicated that TLR2- and TLR4- mediated signaling could enhance IgA immune response during bacterial infection (Li et al., 2019). As shown in Fig. 2A and B, relative expression levels of TLR2 and TLR4 of LPCs stimulated with these LAB were significantly increased compared to those of unstimulated control (control). Subsequently, ELISAs were performed to determine IL-6 and TGF-β levels in culture supernatants from LPCs stimulated with these LAB. Levels of IL-6 were found to be significantly elevated in culture supernatants from LPCs stimulated with these LAB compared to those in unstimulated control LPCs (control; Fig. 2C). However, TGF-β levels in culture supernatants from LPCs stimulated with these LAB were not significantly different from those in unstimulated control LPCs (Fig. 2D). We also monitored expression levels of pIgR in culture supernatants from LPCs stimulated with these LAB. Results indicated that pIgR expression levels in LPCs stimulated with these LAB were not significantly different from those in unstimulated LPCs (Fig. 2E).
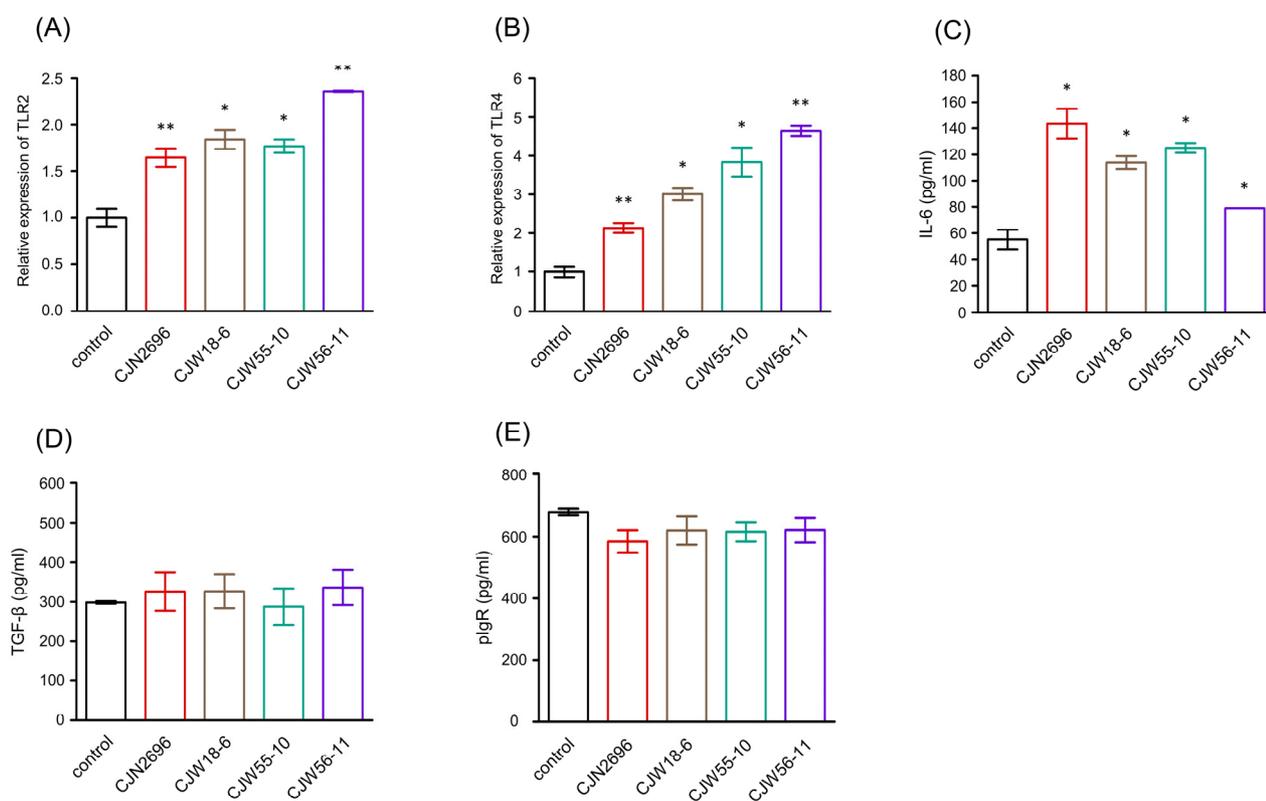
Fig. 2. Selected strains of LAB isolated from traditional fermented Korean foods can increase expression levels of TLR2 and TLR4 and promote the secretion of IL-6 within LPCs. LPCs (2.5×10^5 cells/well) from Peyer's patches of Balb/c mice were co-cultured with each strain of heat-killed bacteria (5×10^6 CFU/mL). (A, B) After 7 days, quantitative real-time PCR analyses were performed with total RNAs isolated from LPCs to detect (A) *Tlr2* and (B) *Tlr4* expressions within LPCs. (C–E) After 7 days, ELISAs were performed with culture supernatant of co-culture to detect (C) IL-6, (D) TGF-β, and (E) pIgR. Control, no treatment. Data are shown as mean±SEM from three independent experiments. Significant differences compared with the control group are indicated by * $p<0.05$ or ** $p<0.01$. TLR, toll-like receptor; IL-6, interleuckin-6; TGF-β, transforming growth factor-β; pIgR, poly Ig receptor; LAB, lactic acid bacteria; LPCs, lamina propria cells; PCR, polymerase chain reaction; ELISAs, enzyme-linked immunosorbent assays.

To determine whether TLR2 or TLR4 signaling affected IgA production in LPCs stimulated by these LAB, LPCs from Peyer's patches of TLR4 null mice (C3H/HeJ) or wild-type TLR4 (C3H/HeN) mice were isolated and stimulated with CJN2696 in the presence of neutralizing antibody against TLR2 (Hagberg et al., 1984). Seven days after co-culture, levels of IgA and the frequency of IgA secreting B cells (IgA$^+$B220$^+$ cells) were measured using ELISAs and flow cytometry analyses, respectively. There was no significant difference in IgA production or frequency of IgA$^+$B220$^+$ cells between CJN2696-stimulated LPCs from C3H/HeJ or those from C3H/HeN mice (Figs. 3A and B). However, anti-TLR2 antibody treatment severely impaired IgA production in both CJN2696-stimulated LPCs from C3H/HeJ and those from C3H/HeN mice (Fig. 3A). Supporting this result, frequencies of IgA$^+$B220$^+$ cells from CJN2696-stimulated LPCs of both C3H/HeJ and C3H/HeN mice were significantly decreased in the presence of anti-TLR2 antibody (Fig. 3B). Therefore, CJN2696 may induce IgA and IL-6 secretion through TLR2.

Oral administration of selected lactic acid bacteria (LAB) increases both mucosal and serum immunoglobulin A (IgA)

Further, we tested whether oral uptake of selected LAB increased IgA production *in vivo*. After 7 days of daily oral administration, IgA levels in GALTs and BALTs of CJN2696, CJW18-6, and CJW55-10 feed animals were elevated compared to those in control mice (Figs. 4A and B). IgA levels in GALTs of these LAB feed mice were comparable between one-week and three-weeks of oral administration (Fig. 4A). Interestingly, IgA levels in BALTs of these LAB feed mice after three-weeks of daily oral administration were slightly decreased compared to those of one-week feed mice (Fig. 4B). Serum IgA levels were increased in CJN2696 feed mice after one-week or three-week administration and in CJW55-10 feed mice after three-week administration compared to those in control mice (Fig. 4C). However, serum IgG levels were not significantly changed before or after administration of these LAB (Fig. 4D). These results indicate that 7 day administration
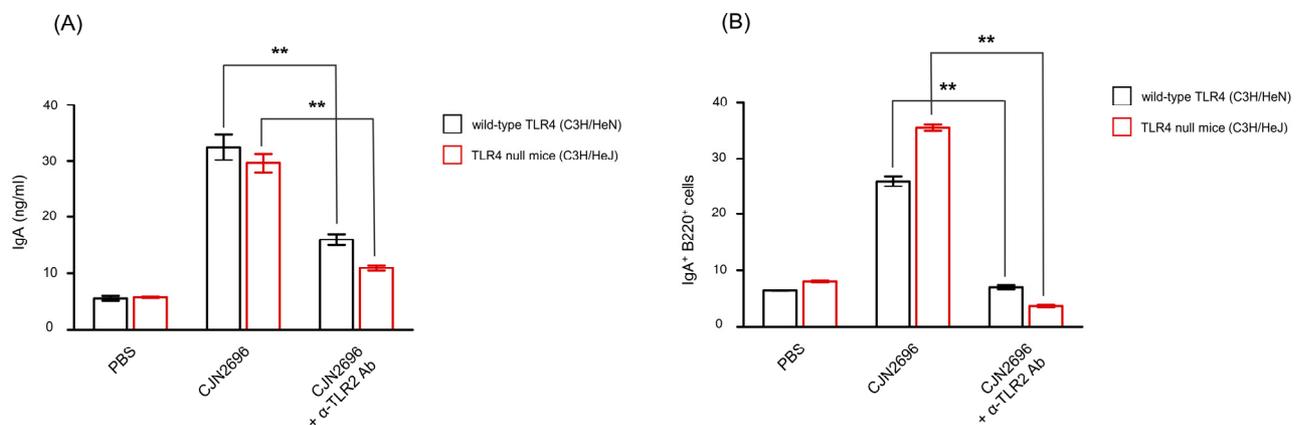
Fig. 3. *Pediococcus acidilactici* CJN2696 promotes IgA secretion of LPCs via TLR2 signaling pathway. LPCs (2.5×10^5 cells/well) from Peyer's patches of wild-type TLR4 (C3H/HeN) mice or TLR4 null mice (C3H/HeJ) were co-cultured with *P. acidilactici* CJN2696 (5×10^6 CFU/mL) in the absence or presence of anti-TLR2 neutralizing antibody (α-TLR2 Ab). After 7 days, (A) ELISAs were performed with culture supernatant of co-culture to detect IgA. (B) Flow cytometry analyses were performed for LPCs to detect IgA positive B cells (IgA$^+$B220$^+$ cells) within LPCs. PBS, LPC culture only; CJN2696, co-culture without antibody; CJN2696+α-TLR2 Ab, co-culture with antibody. Control, no treatment. Data are shown as mean±SEM from three independent experiments. Significant differences between two different groups are indicated by ** p<0.01. IgA, immunoglobulin A; TLR, toll-like receptor; LPCs, lamina propria cells; ELISAs, enzyme-linked immunosorbent assays.
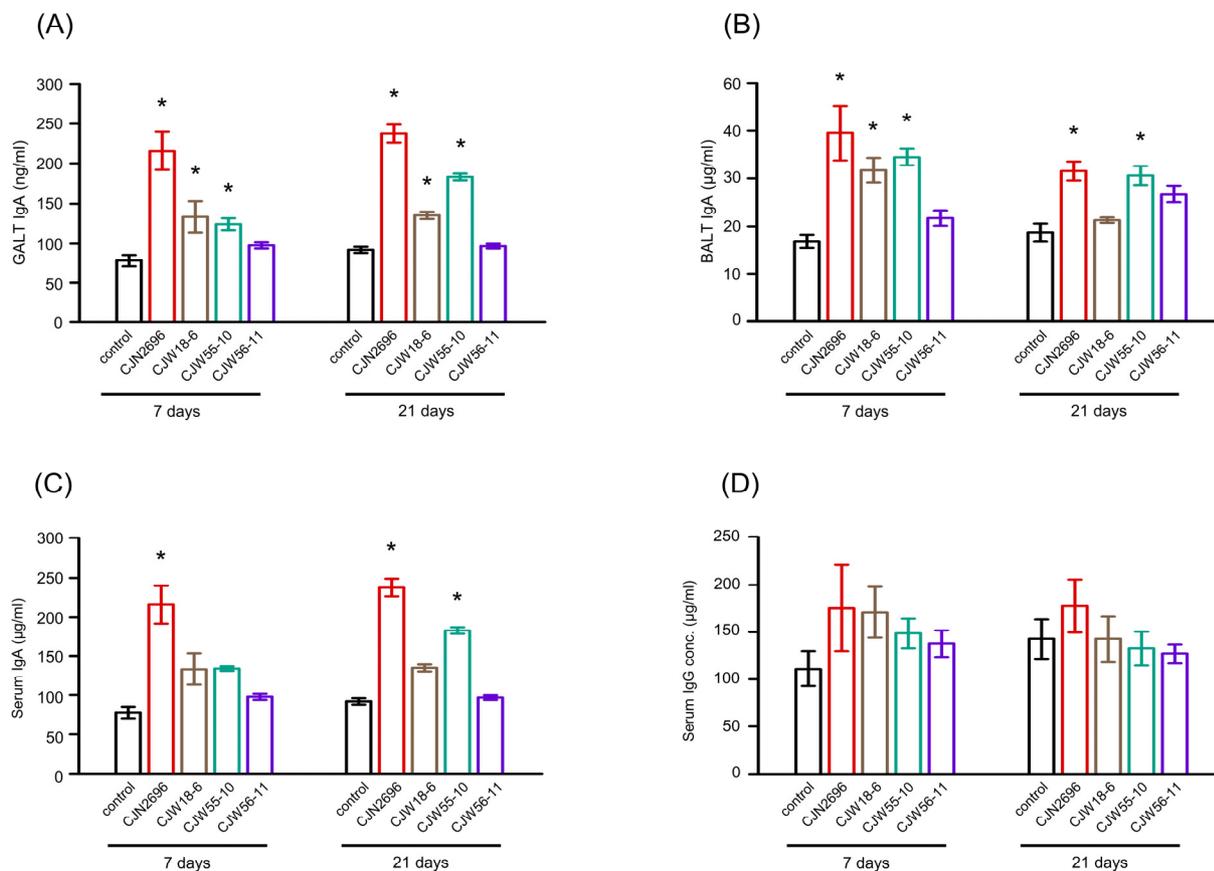
Fig. 4. Oral administration of selected LAB increases systemic IgA secretion. Balb/c mice were randomly separated into ten groups (n=6 mice per group): Two control (PBS) groups and eight test (LAB) groups. For the two control groups, each mouse was orally administered 400 μL of PBS every day for 7 days or 21 days. For the eight test groups, each mouse was orally administered 400 μL of PBS containing LAB (CJW55-10, CJW18-6, CJW56-11, or CJN2696) every day for 7 days or 21 days. At 7 or 21 days after oral administration, mice were sacrificed for analysis. (A) Levels of GALT IgA, (B) levels of BALT IgA, (C) levels of serum IgA, (D) levels of serum IgG. Data are shown as means±SEM (n=6). Significant differences compared with the control group are indicated by * $p<0.05$. GALT, gut-associated lymphoid tissue; IgA, immunoglobulin A; BALT, bronchus-associated lymphoid tissue; LAB, lactic acid bacteria.

of selected LAB is enough to increase not only mucosal IgA, but also serum IgA in mice.

Discussion

In this study, we screened various strains of LAB isolated from traditional fermented Korean foods based on their abilities to enhance IgA production of LPCs from Peyer's patch and measured the efficacy of IgA production after oral administration of selected LAB. Most of these LAB isolated from traditional fermented Korean foods were *Levilactobacillus brevis*, *Lacticaseibacillus casei*, *L. pentosus*, and *L. plantarum*. Since all identified species of LAB are widely used as probiotics, traditional fermented Korean foods might provide a good probiotic repertoire as a functional food supplement to modulate human physiology (De Filippis et al., 2022).

When LPCs from Peyer's patch were co-cultured with these LAB, *L. plantarum* CJW55-10, *L. pentosus* CJW18-6, *L. pentosus* CJW56-11, and *P. acidilactici* CJN2696 induced strong IgA secretions by increased production of IL-6 and elevated number of IgA secreting B cells (IgA+B220+ cells). Among various cytokines, IL-6 is essential for IgA production of mucosal

B cells and TGF-β is involved in mediation of antibody isotype switch to IgA (Ramsay et al., 1994). Especially, IL-6 is indispensable for mucosal IgA production since IL-6 deficient mice exhibit significantly decreased level of IgA with much reduced number of IgA secreting B cells (IgA$^+$B220$^+$ cells) in MALT. However, TGF-β is not essential for the production of IgA since the proper number of IgA secreting B cells (IgA$^+$B220$^+$ cells) were still present in MALT of mice lacking the TGF-β receptor in B cells (Borsutzky et al., 2004). Consistent with these observations, our results demonstrated that only IL-6, but not TGF-β, was increased when LPCs were co-cultured with these LAB. Notably, the proportion of IgA secreting B cells (IgA$^+$B220$^+$ cells) was increased while the proportion of total B cells (B220$^+$ cells) was not changed when LPCs were co-cultured with these LAB. This result might suggest that some molecular patterns derived from these LAB increased the proportion of IgA secreting B cells (IgA$^+$B220$^+$ cells) instead of facilitating the proliferation of whole LP B cells. Supporting this idea, the number of immature B cells (IgM$^+$B220$^+$ cells) in MALT were significantly elevated in IL-6 deficient mice compared to those in wild-type mice (Ramsay et al., 1994). Thus, combined evidence may suggest that IL-6 function is involved in terminal differentiation into IgA secreting B cells, rather than maintenance or proliferation of B cells in MALT (Ramsay et al., 1994).

TLR2 signaling is well-known to trigger host cell responses against Gram-positive bacteria because peptidoglycan and lipoteichoic acid derived from these bacteria are recognized by TLR2 (Schwandner et al., 1999). TLR4 can initiate host responsiveness against Gram-negative bacteria since TLR4 can recognize lipopolysaccharide (Tapping et al., 2000). However, both TLR2 and TLR4 are up-regulated in LPCs stimulated with selected LAB in our study. Previous observation has also indicated that TLR4 signaling may participate in the responsiveness against Gram-positive bacteria *in vitro* (Takeuchi et al., 1999). Interestingly, previous observation showed that TLR4 mRNA expression in human monocytes was substantially decreased by tumor necrosis factor-α treatment, but dramatically increased by IL-6 treatment (Tamandl et al., 2003). Therefore, it is possible that the elevated level of TLR4 expression in LPCs is due to the exposure of IL-6 in the culture supernatant. Since both TLR2 and TLR4 signaling trigger the production of IL-6 through activation of transcription factors such as nuclear factor-kB or interferon-regulatory factors (Zakeri and Russo, 2018), we further examined the effect of TLR2 or TLR4 signaling on the production of IgA by CJN2696 stimulated LPCs. Results clearly indicated that CJN2696 could facilitate IgA and IL-6 production through TLR2. Similar mechanism has been reported for *L. pentosus* b240 in stimulating IgA secretion (Kotani et al., 2014). In addition, it is known that pIgR makes a complex with IgA produced within LP and facilitates a transport of IgA to luminal side of mucosal barrier (Kaetzel, 2005). However, the level of pIgR was not changed when LPCs were co-cultured with these LAB.

Based on our study, *P. acidilactici* CJN2696 is identified as the most powerful IgA stimulant among all LAB tested in this study. *P. acidilactici* is a facultative anaerobic Gram-positive coccus commonly found in fermented vegetables, fermented dairy products, and meat (Papagianni and Anastasiadou, 2009). Although the usage of *P. acidilactici* strain is limited as a human supplementary diet, *P. acidilactici* has been used as an animal feed additive to improve growth performance (Arsène et al., 2021). Also, some strains of *P. acidilactici* can produce pediocin, a powerful bacteriocin that can effectively kill a broad range of pathogenic bacteria (Fugaban et al., 2022). Therefore, selected strains of *P. acidilactici* have the potential as probiotics to improve human health.

In our molecular model, some molecular patterns derived from selected LAB are recognized by pattern recognition receptors such as TLR2 or TLR4. Then, TLR signaling triggers induction of IL-6. Promptly, TLR / IL-6 axis enhance IgA secretion by increasing the proportion of IgA secreting B cells (IgA$^+$B220$^+$ cells). Furthermore, we demonstrated that daily oral intake of selected LAB after 7 days could increase systemic IgA levels including serum and mucosal IgA found in GALT

as well as BALT.

Further study is needed to for structural identification of cell wall components of these LAB for stimulating IgA production and examining protective roles of selected LAB during infection of certain microbial pathogens. Also, additional studies required to monitor changes of gut microbiome populations after the consumption of selected LAB to find out any specific gut microbiome-related linkage for immune modulation. Finally, whole-genome analyses or safety assessment of these LAB needs to be performed because previous studies have clearly shown that LAB even if traditionally were considered as safe, some specific strains can be caring virulence genes and be of potential health hazard (Wang et al., 2021).

Conclusion

In this study, we identified four different strains of LAB which induce IgA secretion by LPCs via TLR / IL-6 signaling pathway in Peyer's patch. Further, we provided evidence that oral administration by these strains of LAB led to increase the levels of GALT IgA, BALT IgA, and serum IgA in mice. Therefore, oral intake of selected strains of LAB can increase systemic IgA secretion in mice.

Conflicts of Interest

Hyun Sun Yun is currently employed by the CJ CheilJedang Corporation (Korea).

Acknowledgements

Author Contributions

Conceptualization: Yun HS, Chun T. Data curation: Choi CY, Lee CH. Formal analysis: Choi CY, Lee CH, Yun HS. Methodology: Choi CY, Lee CH, Yang J, Kang SJ, Park IB, Park SW. Software: Kang SJ, Lee NY, Hwang HB. Validation: Choi CY, Lee CH. Investigation: Chun T. Writing - original draft: Choi CY, Lee CH. Writing - review & editing: Choi CY, Lee CH, Yang J, Kang SJ, Park IB, Park SW, Lee NY, Hwang HB, Yun HS, Chun T.

Ethics Approval

A protocol of animal study was approved by the Institutional Animal Care and Use Committee (IACUC) of Korea University (protocol numbers: KUIACUC-2016-160 and KUIACUC-2017-107).

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