1 **Original research article**

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3 **Efficacies of potential probiotic candidates isolated from traditional fermented**

4 **Korean foods in stimulating immunoglobulin A secretion**

5

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14 **Running headline:** Lactic acid bacteria enhance IgA production

15

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33

34   **Abbreviations:** bronchus-associated lymphoid tissue (BALT), gut-associated lymphoid

35   tissue (GALT), immunoglobulin A (IgA), interleukin (IL), lactic acid bacteria (LAB),

36   lamina propria cell (LPC), poly Ig receptor (pIgR), transforming growth factor-β (TGF-

37   β), toll-like receptor (TLR)

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43 **Original research article**

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57 **Abstract**

58

59   The aim of this study was to evaluate efficacies of selected lactic acid bacteria

60 (LAB) in inducing immunoglobulin A (IgA) secretion. Twenty-five different LAB

61 isolated from traditional fermented Korean foods were characterized for their probiotic

62 properties and screened to identify those that could stimulate lamina propria cells

63 (LPCs) from Peyer's patch to secret IgA *in vitro*. Among them, four strains

64 (*Lactiplantibacillus plantarum* CJW55-10, *Lactiplantibacillus pentosus* CJW18-6, *Lact.*

65 *pentosus* CJW56-11, and *Pediococcus acidilactici* CJN2696) were found to be strong

66 IgA inducers. The number of IgA positive B cells and soluble IgA level were increased

67 when LPCs were co-cultured with these LAB. Expression levels of toll-like receptor

68 (TLR) such as TLR2 and TLR4 and secretion of interleuckin-6 (IL-6) were augmented

69 in LPCs treated with these LAB. Further, we determined whether oral intake of these

70 LAB enhanced IgA production *in vivo*. After one-week of daily oral administration,

71 these LAB feed mice increased mucosal IgA and serum IgA. In conclusion, selected

72 strains of LAB could induce systemic IgA secretion by activating lamina propria B cells

5

73   in Peyer's patch and oral intake of selected strains of LAB can enhance systemic

74   immunity by inducing mucosal IgA secretion.

75

78

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).

95   Recent evidence suggests that administration of certain strain of lactic acid bacteria

96   (LAB) such as *Lacticaseibacillus rhamnosus GG* can facilitate IgA secretion by

97   stimulating immune cells within GALT (Jin et al., 2021). Moreover, IgA induction by

98   *Lactiplantibacillus pentosus* b240 can make mice become more resistant to influenza

99   virus or *Streptococcus pneumoniae* infection (Tanaka et al., 2011; Kiso et al., 2013).

100   These results implied that produced IgA after stimulation with LAB in GALT could

101   protect infection against microbial pathogens through systemic circulation. Therefore,

102   the oral intake of certain LAB might benefit the host through increased protection

103   against certain microbial pathogens by enhancing mucosal immunity. However, most of

104   study describing the enhancement of IgA production by LAB just focused on the

105   efficacy of LAB without any mechanical insights.

106   In this study, we screened 25 different strains of LAB isolated from traditional

107   fermented Korean foods based on their abilities to induce IgA secretion *in vitro* and *in*

108   *vivo*. Results clearly indicated that some of LAB stimulate lamina propria cells (LPCs)

109   from Peyer's patch to secret IgA via toll-like receptor (TLR) / interleuckin-6 (IL-6) axis.

110   Further, we demonstrated that oral stimulation by these LAB increased systemic IgA

111   levels including levels of GALT IgA, bronchus-associated lymphoid tissue (BALT) IgA

112   and serum IgA in mice.

113

Materials and methods

Isolation, identification and characterization of 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 °C 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.

10

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, St. Louis, MO, USA) for 3 h at 37 °C or MRS broth

containing 0.3% oxgall (Sigma) for 24 h at 37 °C. After culture, 100 µl of broth was

plated in triplicate onto MRS agar to measure final CFU.

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)

supplemented with 10% fetal bovine serum (Sigma) 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

146 that attached to HT-29 cells were harvested by repeatedly pipetting with chilled sterile

147 water and cultured on MRS agar plates to measure final CFU. Sources, probiotic

148 properties of identified species and strain names are summarized in Table 1. Each strain

149 of LAB was sub-cultured more than three times prior to *in vitro* experimental analysis.

150

151 **Safety assessment of LAB**

152

153   For measuring hemolytic activity, each strain of LAB was inoculated on BD

154 BBL™ prepared plated medium (Trypticase™ soy agar with 5% sheep blood) (Thermo

155 Fisher Scientific, Waltham, MA, USA) and incubated at 37 °C for 48 h. After 48 h, the

156 colonies on plates were characterized to observe the hemolytic pattern (Fu et al., 2022).

157 To detect urease activity, each strain of LAB was inoculated on Remel™ urea agar base

158 (Thermo Fisher Scientific) and incubated at 37 °C for 48 h. After 48 h, the color

159 changes of plates were monitored to observe the urease activity (Christensen, 1946). For

160 gelatin liquefaction test, each strain of LAB (5.0×10^6 CFU/mL) was cultured in MRS

161 gelatin broth containing 12% (w/v) of gelatin for 48 h at 37 °C. After 48 h, the cultured

162   MRS gelatin broths were incubated at 4 °C to monitor gelatin liquefaction (Fugaban et

163   al., 2021).

164

165   **Animals**

166

167   BALB/c, C3H/HeJ and C3H/HeN mice (female, 6-8 weeks old) were purchased

168   from Central Lab Animal Incorporation (Seoul, Republic of Korea) and acclimated for

169   one week prior to experiments. Mice were fed a standard rodent diet with purified water

170   *ad libitum* and kept at 20-24 °C with 40-60% humidity in Korea University animal

171   facility under a 12 h/12 h light-dark cycle. Mice received proper care in accordance with

172   a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of

173   Korea University (protocol numbers: KUIACUC-2016-160 and KUIACUC-2017-107).

174

175   **Analysis of LAB-stimulated LPCs *in vitro***

176

177   LPCs from Peyer's patches were isolated from the small intestine of BALB/c,

178   C3H/HeJ, or C3H/HeN mouse according to previously established method (Kikuchi et

179   al., 2014). Isolated LPCs (2.5×10^5 cells/well) were then mixed with each heat-killed

180   LAB (5×10^6 CFU/mL) and seeded into 96 well plates in 200 μl of RPMI 1640 medium

181   supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin. After 7 days of

182   incubation, LAB-stimulated LPCs were analyzed for TLR2 and TLR4 expression by

183   quantitative real-time PCR and visualization of IgA positive B cells (IgA^+B220^+ cells)

184   by flow cytometry analysis. Culture supernatant of each sample after co-culture for 7

185   days was harvested. Sandwich ELISAs were performed to detect IgA (Cat # 88-50450-

186   22, Thermo Fisher Scientific, Waltham, MA, USA), IL-6 (Cat # BMS603-2, Thermo

187   Fisher Scientific, Waltham, MA, USA), poly Ig receptor (pIgR) (Cat # SEK50119,

188   SinoBiological, Wayne, PA, USA), and transforming growth factor-β (TGF-β) (Cat #

189   BMS608-4, Thermo Fisher Scientific, Waltham, MA, USA) using ELISA kits according

190   to each manufacturer's instructions. For blocking TLR2 signaling, neutralizing antibody

191   against mouse TLR2 (Cat # 121802, BioLegend, San Diego, CA, USA) was added into

192   the LPC culture.

193

Quantitative real-time PCR

194

195

196   Total RNAs were isolated from cultured lamina propria cells (LPCs) and then

197   cDNAs were synthesized using total RNAs, oligo-dT primers, and a First-Strand cDNA

198   Synthesis Kit (SuperScript RT; Thermo Fisher Scientific). Quantitative real-time PCR

199   was then performed with each cDNA as a template using a QGreen^{TM} $2\times$ qPCR Master

200   Mix (GenDEPOT, Katy, TX, USA) on a Bio-Rad CFX96 Real-Time Detection System

201   (Bio-Rad, Hercules, CA, USA). PCR primer sequences for mouse toll-like receptor 2

202   (*Tlr2*) (GenBank accession NM_011905) were as follows: 5'-

203   TTGCTCCTGCGAACTCCTATCC-3' (sense), 5'-

204   AGTCACACAGGTAGCTGTCTGG-3' (anti-sense), resulting in a RT-PCR product of

205   89 bp in length. PCR primer sequences of mouse *Tlr4* (GenBank accession

206   JX878359.1) were as follows: 5'-GCCGGAAGGTTATTGTGGTAGTG-3' (sense), 5'-

207   GGACAATGA AGATGATGCCAGAGC-3' (anti-sense), resulting in a 123 bp RT-PCR

208   product. Relative expression levels of analyzed genes were normalized to mouse *Gapdh*

209   level. PCR primer sequences of mouse *Gapdh* (GenBank accession NM_008084) were

210 as follows: 5'-ATGGTGAAGGTCGGTGTGAA-3' (sense), 5'-

211 GGTCGTTGATGGCAACAATCTC-3' (anti-sense), resulting in a 100 bp RT-PCR

212 product.

213

214 **Flow cytometry analysis**

215

216 To visualize IgA positive B cells (IgA$^+$B220$^+$ cells), a single cell suspension from

217 cultured LPCs was stained with FITC-conjugated anti-immunoglobulin A (IgA) (Cat #

218 559354, BD Biosciences, San Jose, CA, USA) and PE-conjugated anti-B220 (Cat #

219 553090, BD Biosciences). After staining, cells were washed, resuspended in PBS, and

220 analyzed by flow cytometry using a FACSCalibur with CellQuest software (BD

221 Biosciences) (Choi et al., 2017).

222

223 **Oral administration of LAB and analyses on animal model**

224

225 Sixty Balb/c mice were randomly separated into ten groups (n = 6 mice per group):

226 two control (PBS) groups and eight test (LAB) groups. For the two control groups, each

227 group of mice was orally administered 400 µl of PBS every day for 7 days or 21 days,

228 respectively. For the eight test groups, each group of mice was orally administered 400

229 µl of PBS containing four different strains of LAB (CJW55-10, CJW18-6, CJW56-11,

230 or CJN2696) every day for 7 days or 21 days, respectively. At 7 or 21 days after oral

231 administration, mice were sacrificed to analyze levels of BALT IgA, GALT IgA, serum

232 IgA, and serum IgG using sandwich ELISA kits (Cat # 88-50400-22, Thermo Fisher

233 Scientific, Waltham, MA, USA). For preparing samples of LAB in animal studies, each

234 strain of cultured LAB was freeze-dried and keep at -80 ℃. Each sample of LAB (1 ×

235 10^{10} CFU) was then dissolved in 400 µl of PBS just before oral administration.

236

237 **Analyses of animal model**

238

239   To measure serum IgA and IgG levels in experimental animals, blood specimens

240 from experimental mice were collected from infraorbital veins using capillary tubes on

241 day 7 or 21 after the first oral administration of each strain of LAB. To obtain serum

242   samples, each blood sample was incubated on ice for 1 h and centrifuged at 8,000 g for

243   10 min. Each serum sample was then diluted 1:100 with PBS for measuring IgA and

244   IgG levels (Choi et al., 2017). Sandwich ELISA kits were used to measure total serum

245   IgA (Cat # 88-50450-22, Thermo Fisher Scientific, Waltham, MA, USA) and IgG (Cat

246   # 88-50400-22, Thermo Fisher Scientific) levels according to the manufacturer's

247   instructions.

248   To measure bronchus-associated lymphoid tissue IgA, bronchoalveolar lavage was

249   performed with 2 ml PBS after exposing the tracheae of sacrificed mice.

250   Bronchoalveolar lavage fluids were then centrifuged at 800 g for 5 min at 4℃ (Choi et

251   al., 2018). Levels of IgA in bronchoalveolar lavage fluids were quantitated using

252   sandwich ELISA kits (Cat # 88-50450-22, Thermo Fisher Scientific).

253   To measure gut-associated lymphoid tissue IgA, LPCs from Peyer's patches were

254   isolated from the small intestine of each sacrificed mouse according to previously

255   established method (Kikuchi et al., 2014). Isolated LPCs (2.5×10^5 cells/well) were

256   then mixed with each heat-killed LAB (5×10^6 CFU/ml) and seeded into 96-well plates

257   in 200 μl of RPMI 1640 medium supplemented with 10% (v/v) FBS and 1%

258   penicillin/streptomycin. After 2 days of incubation, culture supernatants of each sample

259   were harvested and sandwich ELISAs were performed to detect IgA levels using ELISA

260   kit (Cat # 88-50450-22, Thermo Fisher Scientific).

261

262   **Statistical analysis**

263

264   Statistical significance of the experimental data was determined by Student's *t*-test.

265   Significant differences at a confidence level of 95% and 99% are labeled with asterisks

266   (*) and (**), respectively, on each graph.

267

268 **Results**

269

270 **Effects of LAB isolated from traditional fermented Korean foods on IgA secretions**

271 **by LPCs *in vitro***

272

273   Twenty-five different strains of LAB were isolated from traditional fermented

274 Korean foods and identified at species level by 16S rDNA sequencing (Table 1).

275 Subsequently, these LAB were characterized for their basic properties as probiotics such

276 as pH tolerance, bile tolerance, and adherence to intestinal epithelial cells (HT-29 cells).

277 All LAB failed to survive or proliferate after three-hour incubation at pH 2.5 (Table 1).

278 However, bile tolerance over 24 h was observed for several strains (Table 1). Also,

279 several strains exhibited adhesive properties to human intestinal epithelial cells (HT-29

280 cells) after three-hour co-culture (Table 1). Next, hemolytic pattern, urease activity and

281 gelatinase activity of these LAB were determined for the preliminary safety evaluation.

282 All the tested strains exhibited γ-hemolytic pattern without activities of both urease and

283 gelatinase (Table 1). To examine immunomodulatory activity, these LAB were

284   examined for their abilities to stimulate LPCs from Peyer's patches to produce IgA.

285   Among them, *Lact. plantarum* CJW55-10, *Lact. pentosus* CJW18-6, *Lact. pentosus*

286   CJW56-11, and *Pediococcus acidilactici* CJN2696 were found to be strong IgA

287   inducers because soluble IgA levels were significantly increased when LPCs were co-

288   cultured with these LAB (Table 1). Supporting this result, IgA secreting B cells

289   (IgA$^+$B220$^+$ cells) were significantly increased when these LAB were co-cultured with

290   LPCs (Fig. 1A). However, total B cells (B220$^+$ cells) of LPCs were not significantly

291   changed after co-culture with these LAB (Fig. 1B).

292

293   **Selected LAB can stimulate IgA and IL-6 secretion through TLR2 signaling**

294

295   To determine how these LAB could stimulate IgA secretion by LPCs, relative

296   expression levels of TLR2 and TLR4 of LPCs stimulated with these LAB were

297   monitored by quantitative real-time PCR analyses because previous observation

298   indicated that TLR2- and TLR4- mediated signaling could enhance IgA immune

299   response during bacterial infection (Li et al., 2019). As shown in Figs. 2A and 2B,

300 relative expression levels of TLR2 and TLR4 of LPCs stimulated with these LAB were

301 significantly increased compared to those of unstimulated control (control).

302 Subsequently, ELISAs were performed to determine IL-6 and TGF-β levels in culture

303 supernatants from LPCs stimulated with these LAB. Levels of IL-6 were found to be

304 significantly elevated in culture supernatants from LPCs stimulated with these LAB

305 compared to those in unstimulated control LPCs (control). (Fig. 2C). However, TGF-β

306 levels in culture supernatants from LPCs stimulated with these LAB were not

307 significantly different from those in unstimulated control LPCs (Fig. 2D). We also

308 monitored expression levels of pIgR in culture supernatants from LPCs stimulated with

309 these LAB. Results indicated that pIgR expression levels in LPCs stimulated with these

310 LAB were not significantly different from those in unstimulated LPCs (Fig. 2E).

311   To determine whether TLR2 or TLR4 signaling affected IgA production in LPCs

312 stimulated by these LAB, LPCs from Peyer's patches of TLR4 null mice (C3H/HeJ) or

313 wild-type TLR4 (C3H/HeN) mice were isolated and stimulated with CJN2696 in the

314 presence of neutralizing antibody against TLR2 (Hagberg et al., 1984). Seven days after

315 co-culture, levels of IgA and the frequency of IgA secreting B cells (IgA$^+$B220$^+$ cells)

316   were measured using ELISAs and flow cytometry analyses, respectively. There was no

317   significant difference in IgA production or frequency of IgA$^+$B220$^+$ cells between

318   CJN2696-stimulated LPCs from C3H/HeJ or those from C3H/HeN mice (Figs. 3A and

319   3B). However, anti-TLR2 antibody treatment severely impaired IgA production in both

320   CJN2696-stimulated LPCs from C3H/HeJ and those from C3H/HeN mice (Fig. 3A).

321   Supporting this result, frequencies of IgA$^+$B220$^+$ cells from CJN2696-stimulated LPCs

322   of both C3H/HeJ and C3H/HeN mice were significantly decreased in the presence of

323   anti-TLR2 antibody (Fig. 3B). Therefore, CJN2696 may induce IgA and IL-6 secretion

324   through TLR2.

325

326   **Oral administration of selected LAB increases both mucosal and serum IgA**

327

328   Further, we tested whether oral uptake of selected LAB increased IgA production

329   *in vivo*. After 7 days of daily oral administration, IgA levels in GALTs and BALTs of

330   CJN2696, CJW18-6, and CJW55-10 feed animals were elevated compared to those in

331   control mice (Figs. 4A and 4B). IgA levels in GALTs of these LAB feed mice were

332   comparable between one-week and three-weeks of oral administration (Fig. 4A).

333   Interestingly, IgA levels in BALTs of these LAB feed mice after three-weeks of daily

334   oral administration were slightly decreased compared to those of one-week feed mice

335   (Fig. 4B). Serum IgA levels were increased in CJN2696 feed mice after one-week or

336   three-week administration and in CJW55-10 feed mice after three-week administration

337   compared to those in control mice (Fig. 4C). However, serum IgG levels were not

338   significantly changed before or after administration of these LAB (Fig. 4D). These

339   results indicate that 7-day administration of selected LAB is enough to increase not only

340   mucosal IgA, but also serum IgA in mice.

341

Discussion

342

343

344   In this study, we screened various strains of LAB isolated from traditional

345   fermented Korean foods based on their abilities to enhance IgA production of LPCs

346   from Peyer's patch and measured the efficacy of IgA production after oral

347   administration of selected LAB. Most of these LAB isolated from traditional fermented

348   Korean foods were *Levilactobacillus brevis*, *Lacticaseibacillus casei*, *Lact. pentosus*

349   and *Lact. plantarum*. Since all identified species of LAB are widely used as probiotics,

350   traditional fermented Korean foods might provide a good probiotic repertoire as a

351   functional food supplement to modulate human physiology (De Filippis et al., 2022).

352   When LPCs from Peyer's patch were co-cultured with these LAB, *Lact. plantarum*

353   CJW55-10, *Lact. pentosus* CJW18-6, *Lact. pentosus* CJW56-11, and *Ped. acidilactici*

354   CJN2696 induced strong IgA secretions by increased production of IL-6 and elevated

355   number of IgA secreting B cells (IgA^+B220^+ cells). Among various cytokines, IL-6 is

356   essential for IgA production of mucosal B cells and TGF-β is involved in mediation of

357   antibody isotype switch to IgA (Ramsay et al., 1994). Especially, IL-6 is indispensable

25

358   for mucosal IgA production since IL-6 deficient mice exhibit significantly decreased

359   level of IgA with much reduced number of IgA secreting B cells (IgA$^+$B220$^+$ cells) in

360   MALT. However, TGF-β is not essential for the production of IgA since the proper

361   number of IgA secreting B cells (IgA$^+$B220$^+$ cells) were still present in MALT of mice

362   lacking the TGF-β receptor in B cells (Borsutzky et al., 2004). Consistent with these

363   observations, our results demonstrated that only IL-6, but not TGF-β, was increased

364   when LPCs were co-cultured with these LAB. Notably, the proportion of IgA secreting

365   B cells (IgA$^+$B220$^+$ cells) was increased while the proportion of total B cells (B220$^+$

366   cells) was not changed when LPCs were co-cultured with these LAB. This result might

367   suggest that some molecular patterns derived from these LAB increased the proportion

368   of IgA secreting B cells (IgA$^+$B220$^+$ cells) instead of facilitating the proliferation of

369   whole LP B cells. Supporting this idea, the number of immature B cells (IgM$^+$B220$^+$

370   cells) in MALT were significantly elevated in IL-6 deficient mice compared to those in

371   wild-type mice (Ramsay et al., 1994). Thus, combined evidence may suggest that IL-6

372   function is involved in terminal differentiation into IgA secreting B cells, rather than

373   maintenance or proliferation of B cells in MALT (Ramsay et al., 1994).

374   TLR2 signaling is well-known to trigger host cell responses against Gram-positive

375   bacteria because peptidoglycan and lipoteichoic acid derived from these bacteria are

376   recognized by TLR2 (Schwandner et al., 1999). TLR4 can initiate host responsiveness

377   against Gram-negative bacteria since TLR4 can recognize lipopolysaccharide (Tapping

378   et al., 2000). However, both TLR2 and TLR4 are up-regulated in LPCs stimulated with

379   selected LAB in our study. Previous observation has also indicated that TLR4 signaling

380   may participate in the responsiveness against Gram-positive bacteria *in vitro* (Takeuchi

381   et al., 1999). Interestingly, previous observation showed that TLR4 mRNA expression

382   in human monocytes was substantially decreased by tumor necrosis factor-α treatment,

383   but dramatically increased by IL-6 treatment (Tamandl et al., 2003). Therefore, it is

384   possible that the elevated level of TLR4 expression in LPCs is due to the exposure of

385   IL-6 in the culture supernatant. Since both TLR2 and TLR4 signaling trigger the

386   production of IL-6 through activation of transcription factors such as nuclear factor-kB

387   or interferon-regulatory factors (Zakeri and Russo, 2018), we further examined the

388   effect of TLR2 or TLR4 signaling on the production of IgA by CJN2696 stimulated

389   LPCs. Results clearly indicated that CJN2696 could facilitate IgA and IL-6 production

390   through TLR2. Similar mechanism has been reported for *Lact. pentosus* b240 in

391   stimulating IgA secretion (Kotani et al., 2014). In addition, it is known that pIgR makes

392   a complex with IgA produced within LP and facilitates a transport of IgA to luminal

393   side of mucosal barrier (Kaetzel, 2005). However, the level of pIgR was not changed

394   when LPCs were co-cultured with these LAB.

395   Based on our study, *Ped. acidilactici* CJN2696 is identified as the most powerful

396   IgA stimulant among all LAB tested in this study. *Ped. acidilactici* is a facultative

397   anaerobic Gram-positive coccus commonly found in fermented vegetables, fermented

398   dairy products, and meat (Papagianni and Anastasiadou, 2009). Although the usage of

399   *Ped. acidilactici* strain is limited as a human supplementary diet, *Ped. acidilactici* has

400   been used as an animal feed additive to improve growth performance (Arsène et al.,

401   2021). Also, some strains of *Ped. acidilactici* can produce pediocin, a powerful

402   bacteriocin that can effectively kill a broad range of pathogenic bacteria (Fugaban et al.,

403   2022). Therefore, selected strains of *Ped. acidilactici* have the potential as probiotics to

404   improve human health.

405   In our molecular model, some molecular patterns derived from selected LAB are

406   recognized by pattern recognition receptors such as TLR2 or TLR4. Then, TLR

407   signaling triggers induction of IL-6. Promptly, TLR / IL-6 axis enhance IgA secretion

408   by increasing the proportion of IgA secreting B cells (IgA$^+$B220$^+$ cells). Furthermore,

409   we demonstrated that daily oral intake of selected LAB after 7 days could increase

410   systemic IgA levels including serum and mucosal IgA found in GALT as well as BALT.

411   Further study is needed to for structural identification of cell wall components of

412   these LAB for stimulating IgA production and examining protective roles of selected

413   LAB during infection of certain microbial pathogens. Also, additional studies required

414   to monitor changes of gut microbiome populations after the consumption of selected

415   LAB to find out any specific gut microbiome-related linkage for immune modulation.

416   Finally, whole-genome analyses or safety assessment of these LAB needs to be

417   performed because previous studies have clearly shown that LAB even if traditionally

418   were considered as safe, some specific strains can be caring virulence genes and be of

419   potential health hazard (Wang et al., 2021).

420

421 **Conclusion**

422

423   In this study, we identified four different strains of LAB which induce IgA

424 secretion by LPCs via TLR / IL-6 signaling pathway in Peyer's patch. Further, we

425 provided evidence that oral administration by these strains of LAB led to increase the

426 levels of GALT IgA, BALT IgA and serum IgA in mice. Therefore, oral intake of

427 selected strains of LAB can increase systemic IgA secretion in mice.

428

429 **Conflict of Interest**

430

431   Yun HS is currently employed by the CJ CheilJedang Corporation, Republic of

432 Korea. None of the other authors had any conflict of interests.

433

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438 **Author Contributions**

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440   Conceptualization: Chun T, Yun HS. Data curation: Choi CY, Lee CH. Formal

441 analysis: Choi CY, Lee CH, Yun HS. Methodology: Choi CY, Lee CH, Yang J, Kang SJ,

442 Park IB, Park SW. Software: Kang SJ, Lee NY, Hwang HB. Validation: Choi CY, Lee

443 CH. Investigation: Chun T. Writing - original draft: Choi CY, Lee CH. Writing - review

444 & editing: Choi CY, Lee CH, Yang J, Kang SJ, Park IB, Park SW, Lee NY, Hwang HB,

445 Yun HS, Chun T.

446

447 **Ethics Approval**

448

449   A protocol of animal study was approved by the Institutional Animal Care and Use

450 Committee (IACUC) of Korea University (protocol numbers: KUIACUC-2016-160 and

451 KUIACUC-2017-107).

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453 **References**

454

455 Arsène MMJ, Davares AKL, Andreevna SL, Vladimirovich EA, Carime BZ, Marouf R,

456   Khelifi I. 2021. The use of probiotics in animal feeding for safe production and as

457   potential alternatives to antibiotics. Vet World 14:319-328.

458 Borsutzky S, Cazac BB, Roes J, Guzmán CA. 2004. TGF-beta receptor signaling is

459   critical for mucosal IgA responses. J Immunol 173:3305-3309.

460 Chen K, Magri G, Grasset EK, Cerutti A. 2020. Rethinking mucosal antibody responses:

461   IgM, IgG and IgD join IgA. Nat Rev Immunol 20:427-441.

462 Choi CY, Kim YH, Oh S, Lee HJ, Kim JH, Park SH, Kim HJ, Lee SJ, Chun T. 2017.

463   Anti-inflammatory potential of a heat-killed *Lactobacillus* strain isolated from

464   Kimchi on house dust mite-induced atopic dermatitis in NC/Nga mice. J Appl

465   Microbiol 123:535-543.

466 Choi SP, Oh HN, Choi CY, Ahn H, Yun HS, Chung YM, Kim B, Lee SJ, Chun T.

467   2018. Oral administration of *Lactobacillus plantarum* CJLP133 and CJLP243

468   alleviates birch pollen-induced allergic rhinitis in mice. J Appl Microbiol 124:821-

469   828.

470   Christensen WB. 1946. Urea decomposition as a means of differentiating *Proteus* and

471   paracolon cultures from each other and from *Salmonella* and *Shigella* types. J

472   Bacteriol 52:461-466.

473   De Filippis F, Esposito A, Ercolini D. 2022. Outlook on next-generation probiotics from

474   the human gut. Cell Mol Life Sci 79:76.

475   Fu X, Lyu L, Wang Y, Zhang Y, Guo X, Chen Q, Liu C. 2022. Safety assessment and

476   probiotic characteristics of *Enterococcus lactis* JDM1. Microb Pathog 163:105380.

477   Fugaban JII, Vazquez Bucheli JE, Park YJ, Suh DH, Jung ES, Franco BDGM, Ivanova

478   IV, Holzapfel WH, Todorov SD. 2022. Antimicrobial properties of *Pediococcus*

479   *acidilactici* and *Pediococcus pentosaceus* isolated from silage. J Appl Microbiol

480   132:311-330.

481   Hagberg L, Hull R, Hull S, McGhee JR, Michalek SM, Svanborg Edén C. 1984.

482   Difference in susceptibility to gram-negative urinary tract infection between

483   C3H/HeJ and C3H/HeN mice. Infect Immun 46:839-844.

484   Jin YB, Cao X, Shi CW, Feng B, Huang HB, Jiang YL, Wang JZ, Yang GL, Yang WT,

485   Wang CF. 2021. *Lactobacillus rhamnosus* GG promotes early B lineage

486   development and IgA production in the lamina propria in Piglets. J Immunol

487   207:2179-2191.

488 Kaetzel CS. 2005. The polymeric immunoglobulin receptor: bridging innate and

489   adaptive immune responses at mucosal surfaces. Immunol Rev 206:83-99.

490 Kawashima T, Ikari N, Kouchi T, Kowatari Y, Kubota Y, Shimojo N, Tsuji NM. 2018.

491   The molecular mechanism for activating IgA production by *Pediococcus*

492   *acidilactici* K15 and the clinical impact in a randomized trial. Sci Rep 8:1-9.

493 Kikuchi Y, Kunitoh-Asari A, Hayakawa K, Imai S, Kasuya K, Abe K, Adachi Y,

494   Fukudome S, Takahashi Y, Hachimura S. 2014. Oral administration of

495   *Lactobacillus plantarum* strain AYA enhances IgA secretion and provides survival

496   protection against influenza virus infection in mice. PLoS One 22:e86416.

497 Kiso M, Takano R, Sakabe S, Katsura H, Shinya K, Uraki R, Watanabe S, Saito H, Toba

498   M, Kohda N, Kawaoka Y. 2013. Protective efficacy of orally administered, heat-

499   killed *Lactobacillus pentosus* b240 against influenza A virus. Sci Rep 3:1-8.

500 Kotani Y, Kunisawa J, Suzuki Y, Sato I, Saito T, Toba M, Kohda N, Kiyono H. 2014.

501 Role of *Lactobacillus pentosus* strain b240 and the toll-like receptor 2 axis in

502 Peyer's patch dendritic cell-mediated immunoglobulin A enhancement. PLoS One

503 9:e91857.

504 Lee J, Yun HS, Cho KW, Oh S, Kim SH, Chun T, Kim B, Whang KY. 2011. Evaluation

505 of probiotic characteristics of newly isolated Lactobacillus spp.: immune

506 modulation and longevity. Int J Food Microbiol 148:80-86.

507 Li X, Zhang YK, Yin B, Liang JB, Jiang F, Wu WX. 2019. Toll-like receptor 2 (TLR2)

508 and TLR4 mediate the IgA immune response induced by *Mycoplasma*

509 *hyopneumoniae*. Infect Immun 88:e00697-19.

510 Mörbe UM, Jørgensen PB, Fenton TM, von Burg N, Riis LB, Spencer J, Agace WW.

511 2021. Human gut-associated lymphoid tissues (GALT); diversity, structure, and

512 function. Mucosal Immunol 14:793-802.

513 Papagianni M, Anastasiadou S. 2009. Pediocins: The bacteriocins of *Pediococci*.

514 Sources, production, properties and applications. Microb Cell Fact 8:1-16.

515 Ramsay AJ, Husband AJ, Ramshaw IA, Bao S, Matthaei KI, Koehler G, Kopf M. 1994.

516 The role of interleukin-6 in mucosal IgA antibody responses *in vivo*. Science

517 264:561-563.

518   Schwandner R, Dziarski R, Wesche H, Rothe M, Kirschning CJ. 1999. Peptidoglycan-

519   and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. J

520   Biol Chem 274:17406–17409.

521   Swain S, Selmi C, Gershwin ME, Teuber SS. 2019. The clinical implications of

522   selective IgA deficiency. J Transl Autoimmun 2:100025.

523   Takahashi D, Kimura S, Hase K. 2021. Intestinal immunity: to be, or not to be,

524   induced? That is the question. Int Immunol 33:755-759.

525   Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, Takeda K, Akira S.

526   1999. Differential roles of TLR2 and TLR4 in recognition of gram-negative and

527   gram-positive bacterial cell wall components. Immunity 11:443–451.

528   Tamandl D, Bahrami M, Wessner B, Weigel G, Ploder M, Fürst W, Roth E, Boltz-

529   Nitulescu G, Spittler A. 2003. Modulation of toll-like receptor 4 expression on

530   human monocytes by tumor necrosis factor and interleukin-6: tumor necrosis

531   factor evokes lipopolysaccharide hyporesponsiveness, whereas interleukin-6

532   enhances lipopolysaccharide activity. Shock 20:224-229.

533   Tanaka A, Seki M, Yamahira S, Noguchi H, Kosai K, Toba M, Morinaga Y, Miyazaki T,

534   Izumikawa K, Kakeya H, Yamamoto Y, Yanagihara K, Tashiro T, Kohda N, Kohno

535   S. 2011. *Lactobacillus pentosus* strain b240 suppresses pneumonia induced by

536   *Streptococcus pneumoniae* in mice. Lett Appl Microbiol 53:35-43.

537   Tapping RI, Akashi S, Miyake K, Godowski PJ, Tobias PS. 2000. Toll-like receptor 4,

538   but not toll-like receptor 2, is a signaling receptor for *Escherichia* and *Salmonella*

539   lipopolysaccharides. J Immunol 16:5780–5787.

540   Wang Y, Liang Q, Lu B, Shen H, Liu S, Shi Y, Leptihn S, Li H, Wei J, Liu C, Xiao H,

541   Zheng X, Liu C, Chen H. 2021. Whole-genome analysis of probiotic product

542   isolates reveals the presence of genes related to antimicrobial resistance, virulence

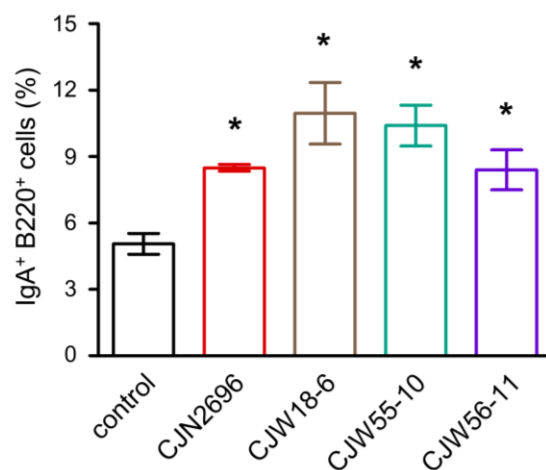543   factors, and toxic metabolites, posing potential health risks. BMC Genomics

544   22:210.

545   Zakeri A, Russo M. 2018. Dual role of toll-like receptors in human and experimental
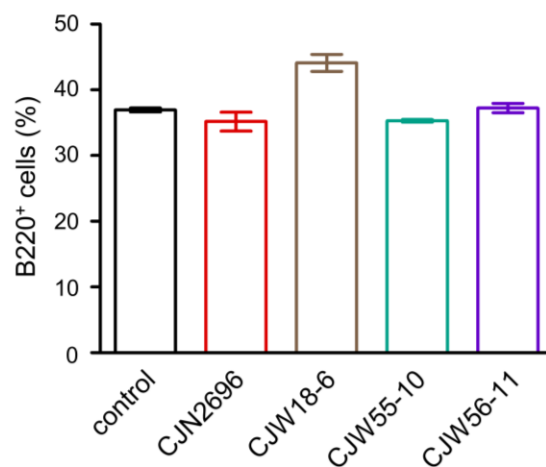
546   asthma models. Front Immunol 9:1027.

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Figure legends

(A)

(B)

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550 **Fig. 1. Selected strains of LAB isolated from traditional fermented Korean foods**

551 **can increase the frequency of IgA positive B cells within LPCs.** LPCs (2.5×10^5 cells

552   well^{-1}) from Peyer's patches of Balb/c mice were co-cultured with each strain of heat-

553   killed bacterium (5×10^6 CFU ml^{-1}). After 7 days, flow cytometry analyses were

554   performed with LPCs to detect (A) IgA positive B cells (IgA$^+$B220$^+$ cells) and (B) total

555   B cells (B220$^+$ cells) within LPCs. Control, no treatment. Data are shown as mean ±

556   SEM from three independent experiments. Significant differences compared with the

557   control group are indicated by *, $P < 0.05$.
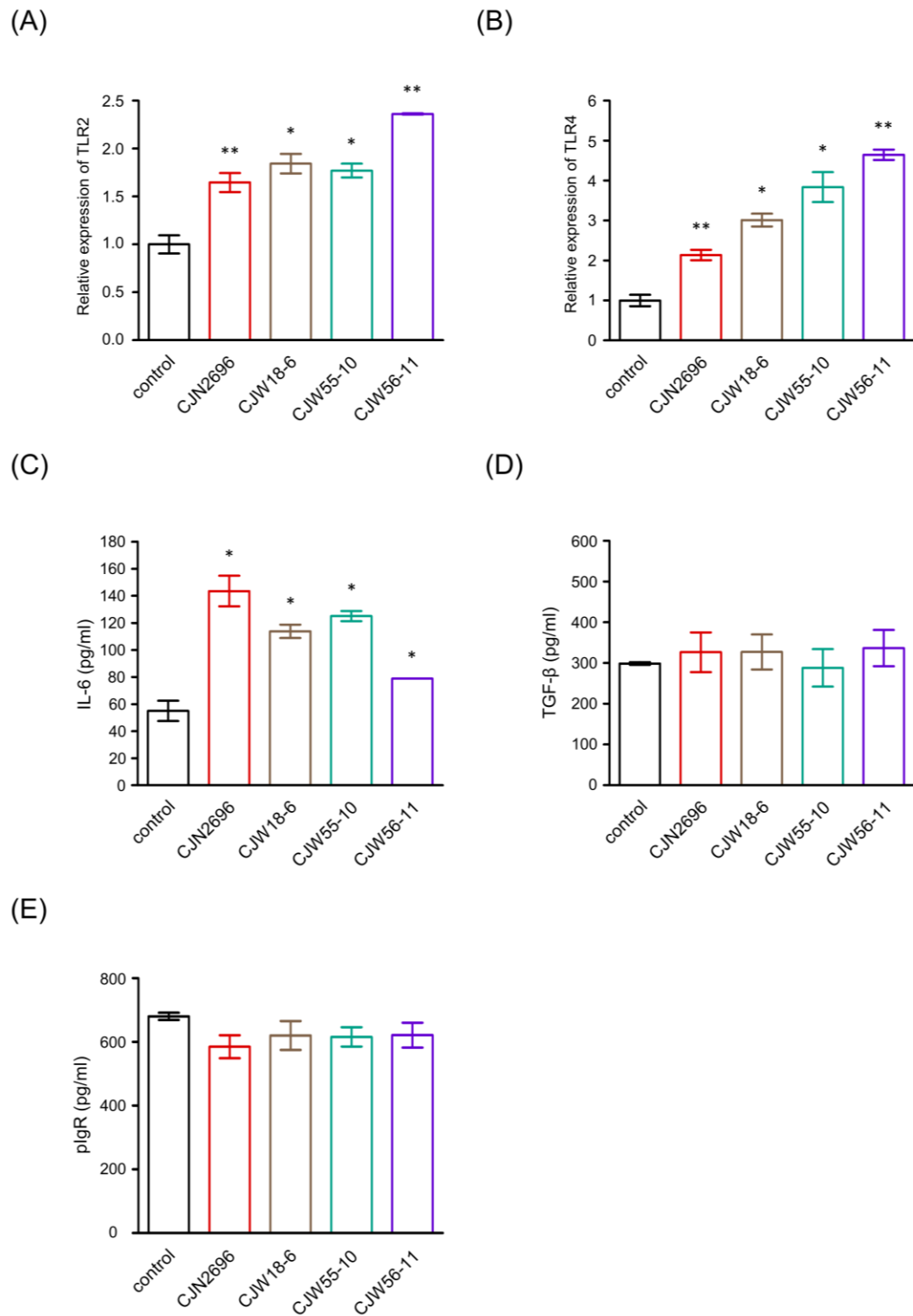
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(A)

(B)

(C)

(D)

(E)

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Fig. 2. Selected strains of LAB isolated from traditional fermented Korean foods

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can increase expression levels of TLR2 and TLR4 and promote the secretion of IL-

6 within LPCs. LPCs (2.5×10^5 cells well^{-1}) from Peyer's patches of Balb/c mice were

co-cultured with each strain of heat-killed bacteria (5×10^6 CFU ml^{-1}). (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 **, $P < 0.01$.

(A)

(B)

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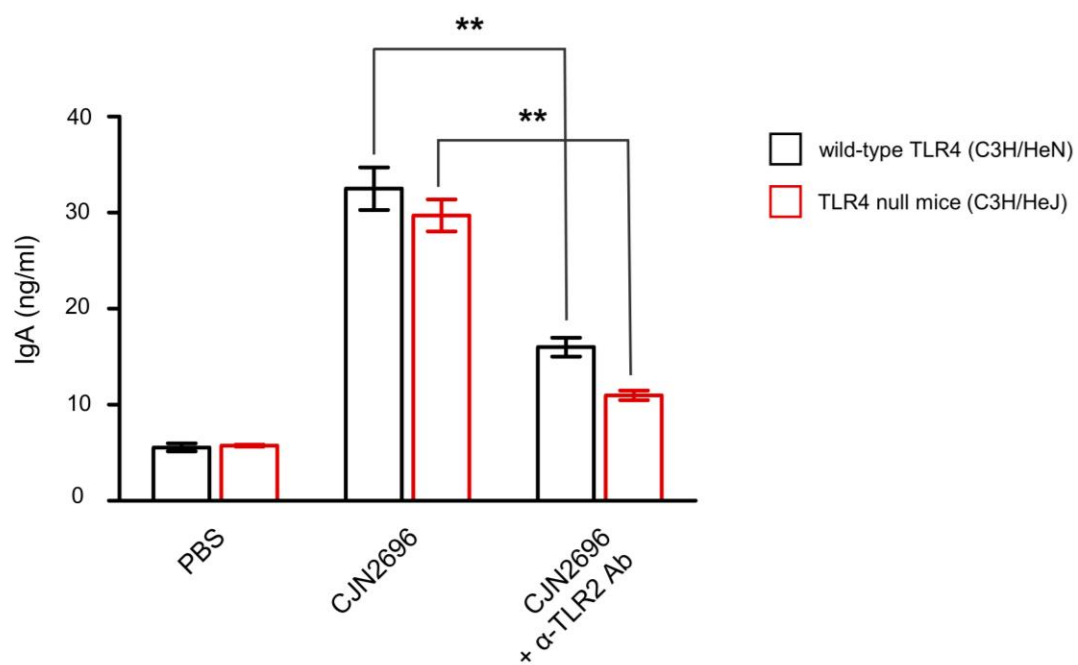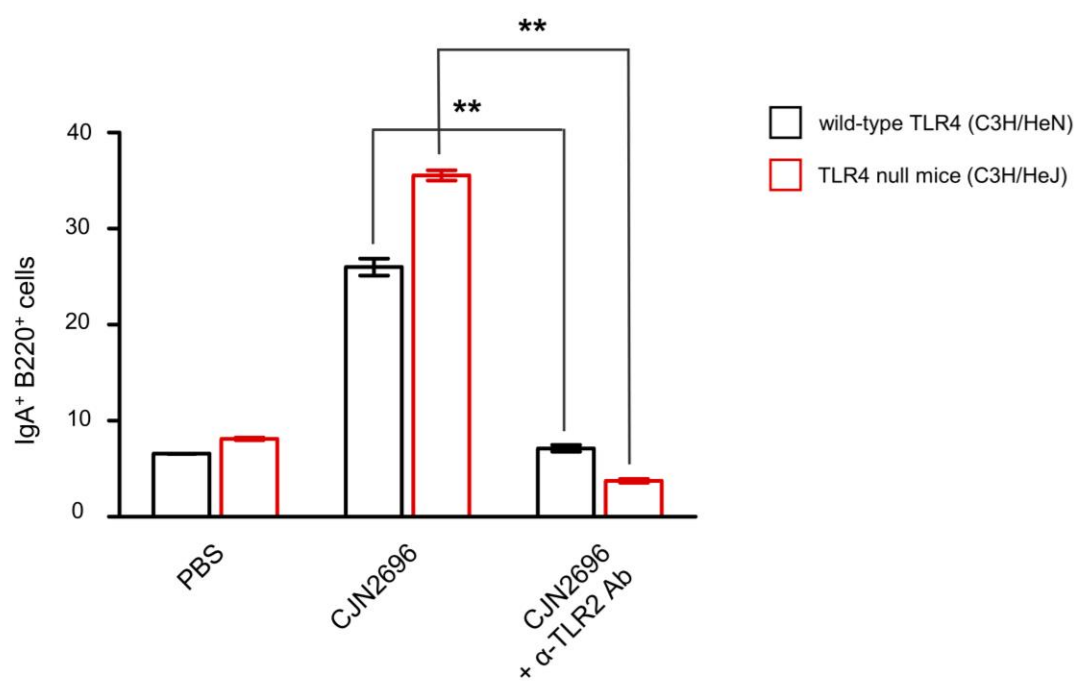
Fig. 3. *Pediococcus acidilactici* **CJN2696 promotes IgA secretion of LPCs via TLR2**

573   **signaling pathway.** LPCs (2.5×10^5 cells well^{-1}) from Peyer's patches of wild-type

574   TLR4 (C3H/HeN) mice or TLR4 null mice (C3H/HeJ) were co-cultured with *Ped.*

575   *acidilactici* CJN2696 (5×10^6 CFU ml^{-1}) in the absence or presence of anti-TLR2

576   neutralizing antibody (α-TLR2 Ab). After 7 days, (A) ELISAs were performed with

577   culture supernatant of co-culture to detect IgA. (B) Flow cytometry analyses were

578   performed for LPCs to detect IgA positive B cells (IgA$^+$B220$^+$ cells) within LPCs. PBS,

579   LPC culture only; CJN2696, co-culture without antibody; CJN2696 + α-TLR2 Ab, co-

580   culture with antibody. Control, no treatment. Data are shown as mean ± SEM from three

581   independent experiments. Significant differences between two different groups are

582   indicated by **, $P < 0.01$.
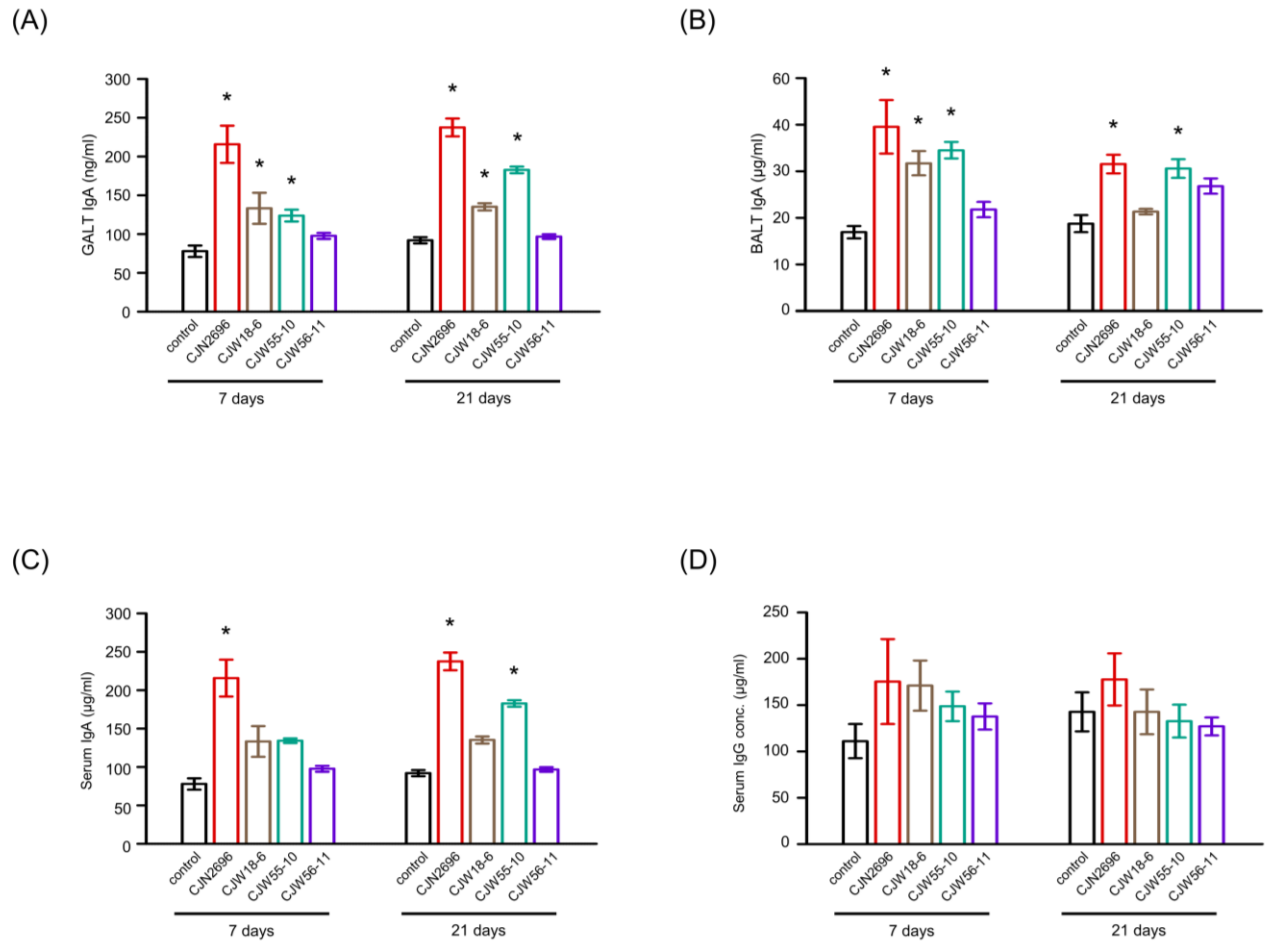
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(A) (B) (C) (D)

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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

47

591   or 21 days after oral administration, mice were sacrificed for analysis. (A) Levels of

592   GALT IgA, (B) Levels of BALT IgA, (C) Levels of serum IgA, (D) Levels of serum

593   IgG. Data are shown as means ± SEM ($n = 6$). Significant differences compared with

594   the control group are indicated by *, $P < 0.05$.

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

Strain	Species[a]	Growth/survival[b]		Adhesion (%)[c]	Hemolytic pattern	Urease activity	Gelatinase activity	IgA secretion (ng/ml)[d,e]	Source
		pH 2.5	Oxgall 0.3%						
CJT1088	*Lactiplantibacillus plantarum*	negative	negative	83	gamma	negative	negative	34 ± 20.8	Doenjang (fermented soybean paste)
NR02	*Lactiplantibacillus plantarum*	negative	negative	84	gamma	negative	negative	93 ± 24.2	Kimchi
CJIH53	*Lactiplantibacillus plantarum*	negative	positive	61	gamma	negative	negative	99 ± 28.7	Kimchi
CJT1085	*Lactiplantibacillus 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	*Lactiplantibacillus 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	*Lacticaseibacillus casei*	negative	positive	77	gamma	negative	negative	47 ± 3.5	Kimchi
CJN1917	*Lactiplantibacillus plantarum*	negative	negative	64	gamma	negative	negative	61 ± 9.5	Maggeolli (rice wine)
CJN2694	*Lacticaseibacillus 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	*Lactiplantibacillus plantarum*	negative	positive	82	gamma	negative	negative	143 ± 54.1	Kimchi
CJW15-5	*Lactiplantibacillus pentosus*	negative	positive	60	gamma	negative	negative	107 ± 7.3	Kimchi
CJW18-3	*Lactiplantibacillus plantarum*	negative	positive	68	gamma	negative	negative	101 ± 5.6	fermented green tea
CJW26-3	*Lactiplantibacillus pentosus*	negative	positive	70	gamma	negative	negative	99 ± 11.3	Kimchi
CJW41-7	*Lactiplantibacillus plantarum*	negative	positive	64	gamma	negative	negative	106 ± 2.8	Kimchi
CJW54-15	*Companilactobacillus farciminis*	negative	positive	ND[f]	gamma	negative	negative	86 ± 1.4	Kimchi
CJW55-1	*Lactiplantibacillus pentosus*	negative	positive	69	gamma	negative	negative	105 ± 7.3	Kimchi

CJW56-3	*Lactiplantibacillus pentosus*	negative	positive	90	gamma	negative	negative	100 ± 13.0	Kimchi
CJN2359	*Lactiplantibacillus 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	*Lactiplantibacillus plantarum*	negative	positive	88	gamma	negative	negative	87 ± 1.1	Kimchi
CJW55-8	*Lactiplantibacillus plantarum*	negative	positive	68	gamma	negative	negative	99 ± 3.9	Kimchi
CJW56-11	*Lactiplantibacillus pentosus*	negative	positive	70	gamma	negative	negative	124 ± 7.3	Kimchi

595   [a]16s rDNA sequenced having over 99% homology.

596   [b]Positive, growth or survival; negative, no growth or survival compared with initial inoculums. Initial inoculums at approximately 1×10^8 CFU/ml (log 8.0).

597   [c]Percentage of adhering lactobacilli in HT-29 cell after 3 h incubation. Initial inoculums at approximately 1×10^8 CFU/ml (log 8.0).

598   [d]Results were shown as means ± standard errors.

599   [e]IgA secretion without LAB stimulation was 22 ± 3.0

600   [f]ND, not determined

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602