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Use of Murine Models To Detect the Allergenicity of Genetically Modified *Lactococcus lactis* NZ9000/pNZPNK

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ABSTRACT: By introducing aprN into *Lactococcus lactis* NZ9000, the genetically modified *L. lactis* NZ9000/pNZPNK successfully expressed the nattokinase. The safety assessment of this novel strain was based on allergenicity of pepsin digestion stability and murine model serologic identity. Subjecting to the GM strain and host to pepsin digestion, the soluble fractions and cell debris were fast degraded completely. Feeding with ovalbumin resulted in significantly higher production of IgG1 and IgE as compared to that of *L. lactis* NZ9000/pNZPNK or *L. lactis* NZ9000. Further, the serum IgG2a level increased dose-dependently at week 2 and induced immune reaction toward Th1 pathway. Secretion of cytokines IL-4 and IL-10 fed with lactococci was significantly lower than that of the OVA group. *L. lactis* NZ9000/pNZPNK did not increase the proliferation of type 2 helper T cells in spleen or induce allergenicity in BALB/c mice. On the basis of the results, the new GM lactic acid bacterium is regarded as safe to use.

KEYWORDS: Genetically modified lactic acid bacteria, *Lactobacillus lactis* NZ9000/pNZPNK, pepsin digestion assay, allergenicity assessment

INTRODUCTION

Genetically modified microorganisms (GMMs) are strains whose genetic makeup is altered by modern biotechnological techniques to produce novel foods. GMMs and their products are intended for human or animal consumption in many fields, such as foods, feeds, and pharmaceutical products.[1−3] Microbial products used in foods may contain viable cells during production or consumption. It is important to ensure that the recipient microorganisms are not pathogenic, toxigenic, or allergenic and will not compromise the safety and nutritional status of foods.[4]

Recently, GMMs including genetically modified (GM) lactic acid bacteria (LAB) have become popular in the field of genetic engineering. However, their safety and efficacy with regard to humans have not yet been defined. Potential allergenicity, in particular, is an important issue that should be addressed in studies reporting on the safety assessment of GMM in humans. There are many evaluation methods that can effectively identify the allergenic potency of the novel GM proteins or products.[1,2] Generally, the methods include the description of source protein, the history of exposure and safety of the gene(s) source, amino acid sequence identity to human allergens, stability to pepsin digestion in vitro, protein abundance in the food and processing effects, and, when appropriate, specific IgE binding studies or skin-prick testing.[2,5]

The pepsin digestion assay is a useful method to evaluate the stability of a novel protein exposed to pepsin protease and extreme pH in a simulated gastric environment.[6,7] In vitro digestibility methods maybe show the existence of digestion-resistant proteins from allergenic food extracts, but not from nonallergenic sources. Animal models may be a selective method to evaluate the serum-specific IgE in the screening of target proteins for allergenic potency in vivo. The main advantages of rodent models are that they are inexpensive and accessible as compared to other animals due to their small size, have a well-characterized immune system, and allow many administration routes such as oral administration, intraperitoneal injection, intragastric gavage, and dermal administration without requiring adjuvant.[5,8−12]

Many LABs are residential microflora in the gastrointestinal tract of mammals. They have been used in fermented dairy or vegetable foods and are categorized as "generally recognized as safe". *Lactococcus lactis*, a gram-positive, nonpathogenic LAB, is one of the most suitable hosts for secretion of heterologous proteins.[13] Nattokinase (NK), also known as serine protease, is an important enzyme for the characteristic taste and flavor of natto; it is a plasminogen activator and shows potent fibrinolytic activity in *Bacillus subtilis* natto in vivo and in vitro tests.[14−17] Fujita et al.[14] proved that NK could pass the rat intestinal tract and it could dissolve chemically induced thrombosis. Tissue-type plasminogen activator and urokinase are widely used in thrombolytic therapy, but they are expensive and have undesirable side effects. Finding cheaper and safer alternatives is a major task for researchers.[18] The microbial fibrinolytic enzymes, such as NK from *Bacillus natto*, attracted much medical interest during the latest decades.[17,19] Ingestion of GM-LAB provides not only the probiotic effects but also the fibrinolytic benefits of microbial enzymes. In our previous study, we constructed a nisin-induced *L. lactis* NZ9000/pNZPNK, which was constructed to combine two potential probiotics: *L. lactis* and the nattokinase (from *Bacillus subtilis* natto). This strain could produce intracellular

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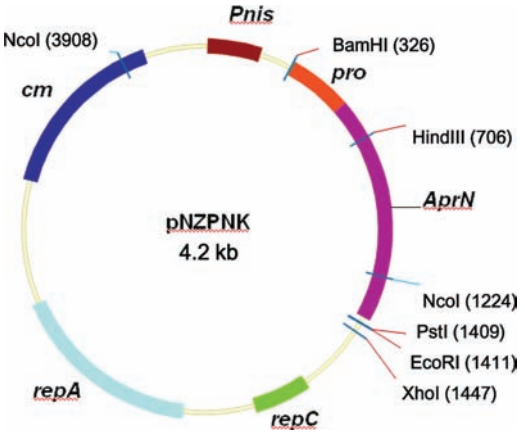

Figure 1. Construction of pNZPNK in *L. lactis* NZ9000/pNZPNK.

NK, suggesting that it could be safely used in animals on the basis of the interaction between test strains and intestinal microflora.[20] However, the allergenicity of NK or total excretion proteins from GM *L. lactis* NZ9000/pNZPNK should be investigated. Our aim was to use it as a model GMM strain to evaluate the allergenicity of the strain by the assessment of in vitro pepsin digestion stability and serologic identity in the mouse model.

MATERIALS AND METHODS

Construction of Plasmids and Incubation Conditions. Primers PNK268F (GATATAGGATCCGCGCAATCTGTTCCTTA-TGGCATTTCT) and NK1326R (GATATACTCGAGTGTACGTT-GATTAACCCTTTTCCATAGTAGAA) were used, which contained the sequence of the *apr*N gene.[15] In the *apr*N gene, the pre, pro, and mature sequences are located at 181−268 bp, 269−498 bp, and 499−1323 bp, respectively. Both primers were designed to introduce *Bam*HI and *Xho*I restriction sites (underlined in the primer sequences) upstream and downstream of the *apr*N gene coding region, respectively. A PCR product with the expected size (1 kb) was obtained and cloned as a *Bam*HI−*Xho*I fragment into the similarly digested, high-copy-number shuttle vector pNZ8020 (NIZO Food Research, The Netherlands) under the control of the *nis*A promoter. The ligation mixture was transformed into *E. coli* JM109, and a recombinant plasmid carrying the appropriate insert (designated pNZPNK) was selected (Figure 1). The pNZPNK plasmid was independently introduced by electroporation into *L. lactis* NZ9000. The strains were cultured in M17 broth supplemented with 0.5% glucose, and a 1% inoculum was subcultured for 18 h at 30 °C. Next, *L. lactis* NZ9000/pNZPNK was induced with nisin to produce NK (1 ng/mL) when the OD_{600} of the medium was 0.4. After 18 h of incubation, the bacterial cells of non-GM *L. lactis* NZ9000 and GM *L. lactis* NZ9000/pNZPNK were centrifuged at 7000*g* for 15 min, washed three times with phosphate buffered saline (PBS), and collected for further test.

In Vitro Pepsin Digestibility and Stability Assay. The pepsin digestibility and stability assay were modified from the method of Thomas et al.[6,7] The pellet of *L. lactis* NZ9000/pNZPNK was dissolved in 5 mL of PBS and disrupted using the cell disruptor One Shot model (Constant Systems, Northants, UK) at 25 kpsi. The suspension was centrifuged at 7000*g* for 15 min. The supernatant was freeze-dried, and its protein concentration was measured using the BCA protein assay reagent (Pierce, Rockford, IL). Bovine serum albumin (BSA) and soybean trypsin inhibitor (STI) were used as sensitive and resistant controls, respectively. A stimulated gastric fluid (SGF) tube, containing 0.084 N HCl, 35 mM NaCl, and 6520 U of pepsin (pH 1.2) in a total volume of 2.5 mL for each protein, was preheated at 37 °C and mixed with 400 μg of test protein solution. The mixtures were placed in a 37 °C water bath, and 200 μL of mixtures was removed at 0.5, 2, 5, 10, 30, and 60 min after the initial reaction. The mixture from each time interval was quenched by adding 70 μL of 200 mM $NaHCO_3$ (pH 11) and 70 μL of 5× Laemmli buffer (40% glycerol, 5% β-mercaptoethanol, 10% SDS, 0.33 M Tris, 0.05% bromophenol blue, pH 6.8) and heating at 100 °C for 10 min. Fifty microliters of the mixtures was subjected to 12.5% SDS-PAGE or Tricine-SDS-PAGE electrophoresis analysis; the gels were stained with 0.1% Coomassie brilliant blue R-250 (Sigma-Aldrich, St Louis, MO) for 10 min and destained in 25% methanol with 7.5% glacial acetic acid.

Western Blotting Analysis. Destained SDS-PAGE or Tricine-SDS-PAGE gels were electrically transferred onto a polyvinylidene difluoride membrane. Next, the membrane was washed twice with PBS containing 0.05% Tween-20 (PBST) and blocked with 5% skim milk in PBS for 2 h. The membrane was incubated with 1:2000 diluted anti-NK antiserums raised against rat (prepared from our laboratory) in PBST at 4 °C overnight. After being washed with PBST three times, the membrane was incubated with 1:5000 diluted ImmunoPure goat antirat IgG conjugated with horseradish peroxidase (HRP) (Pierce) for 1 h, and the washing step was repeated three times. The band for NK was visualized using the Immobilon Western Kit (Millipore, Billerica, MA) and was quantified by an enhanced chemiluminescence detection system (UVP GelDoc-It, Upland, CA).

Animal Models for Oral Exposure. The allergenicity safety of *L. lactis* NZ9000/pNZPNK and *L. lactis* NZ9000 was investigated by feeding trials. The animals were randomly divided into eight groups, each consisting of seven 6−8-week-old female BALB/c mice obtained from the National Laboratory Animal Center, Taipei, Taiwan. Each group was subjected to one of the following eight treatments per day by oral gavage for 6 weeks: (1) 0.25 mL of PBS per day (control, CON), (2) 1× low dosage of *L. lactis* NZ9000 (2.5×10^8 CFU/mouse, NZL), (3) 2× mederate dosage of *L. lactis* NZ9000 (5×10^8 CFU/mouse, NZM), (4) 5× high dosage of *L. lactis* NZ9000 (1.25×10^9 CFU/mouse, NZH), (5) 1× dosage of *L. lactis* NZ9000/pNZPNK (2.5×10^8 CFU/mouse, PNL), (6) 2× dosage of *L. lactis* NZ9000/pNZPNK (5×10^8 CFU/mouse, PNM), (7) 5× dosage of *L. lactis* NZ9000/pNZPNK (1.25×10^9 CFU/mouse, PNH), and (8) 20 mg of ovalbumin (OVA, albumin from chicken egg white, grade III, minimum 90% purified, Sigma-Aldrich). Each dosage of the two strains and OVA were dissolved in 0.25 mL of PBS. The experimental design is outlined in Figure 2. The mice were housed in polycarbonate cages, maintained on a 12 h:12 h light/dark cycle, and given access to food (LabDiet 5001 Rodent diet, chemical compositions: protein 23.9%; fat 5.0%; nitrogen-free extract 48.7%; fiber 5.1%; and gross energy 4.07 kcal/g, Purina Mills LLC, St. Louis, MO) and water ad libitum. This diet is formulated using the unique and innovative concept of Constant Nutrition, paired with the selection of highest quality ingredients to ensure minimal inherent biological variation in long-term studies. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of National Chung-Hsing University, Taichung, Taiwan.

Blood Collection and Preparation of Spleen Cells. Mice were anesthetized with tribromoethanol (Avertin, 250 mg/kg), and blood samples were collected via the retro-orbital sinus at various time periods. After whole blood clotting at room temperature for 2 h, serum was separated by centrifugation (1500*g* for 15 min) and stored at −80 °C until analysis. At the end of the test, the mice were sacrificed and their spleens were removed. After depletion of erythrocytes, splenocytes (1×10^6 cells/100 μL per well) were cultured with or without lipopolysaccharide (LPS, 10 μg/mL; Sigma) or concanavalin A (Con A, 25 μg/mL; Sigma) in RPMI (Roswell Park Memorial Institute)-1640 medium (HyClone, South Logan, UT) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Grand Island, NY) in a 24-well plate at 37 °C in a 5% CO_2

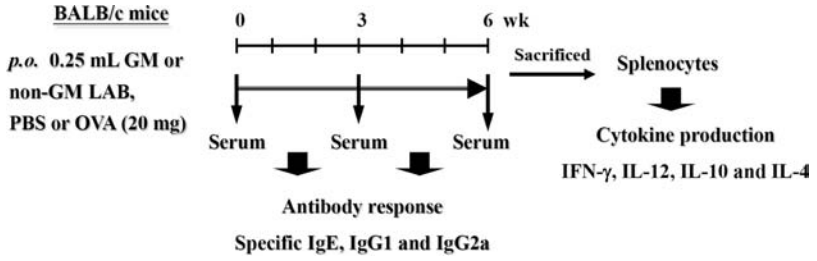

Figure 2. Experimental outline of oral administration of GM *L. lactis* NZ9000/pNZPNK or non-GM *L. lactis* NZ9000 in the murine model.

humidified incubator for 48 h. Next, the plates were centrifuged at 400*g* for 10 min, and the supernatants were collected for cytokine analysis.

Splenocyte Proliferation Assay. Splenocyte proliferation was assayed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT)-based colorimetric method.[21] Splenocytes (2×10^5 cells/100 μL per well) were cultured with or without LPS (Sigma) or Con A (Sigma) in RPMI-1640 medium (HyClone) as previously described in a 96-well plate for 48 h. The plates were incubated at 37 °C in a 5% CO_2 humidified incubator. After incubation for 44 h, 50 μL of MTT solution (5 mg/mL, Sigma) was added to each well and left to stand in the dark for 4 h. The plates were centrifuged at 400*g* for 10 min, and untransformed MTT was carefully removed. Fifty microliters of dimethyl sulfoxide (Sigma) was added to dissolve the insoluble purple formazan product into a colored solution, and the mixture was vigorously shaken for 30 s. The absorbance was measured at 570 nm using a Bio-Rad microplate reader (San Diego, CA). The proliferation index was calculated by dividing the absorbance value of the mitogen-induced culture by the absorbance of the nonstimulated culture.

Measurement of Cytokine and Antigen-Specific IgE, IgG1, and IgG2a Contents by ELISA. IFN-γ, IL-4, IL-10, or IL-12 in the supernatants of splenocytes cell culture was quantified by ELISA using DuoSet mouse IFN-γ, IL-4, IL-12, or IL-10 kits (R&D Systems, Abingdon, UK), respectively. The plates were coated overnight at 4 °C with 100 μL of 1 μg/mL rat antimouse capture antibodies. After being washed three times with PBST, the plates were incubated for 1 h at room temperature with 200 μL of 0.01 M PBS containing 1% (w/v) BSA (Sigma) and subsequently washed three times with PBST. Standard cytokines or samples (100 μL) were added to each well, and the plates were incubated for 1 h at 37 °C. The plates were washed four times with PBST and incubated for 1 h with 100 μL of 500 ng/mL biotinylated rat antimouse detection antibody and then washed four times with PBST. Streptavidin-HRP conjugate (100 μL, Pierce) was added to each well, and the plates were incubated for 30 min and washed five times in PBST. Bound peroxidase conjugate was detected with the addition of 100 μL of tetramethylbenzidine and hydrogen peroxide solution (Pierce). The reaction was halted by the addition of 50 μL of 1 M sulfuric acid, and the absorbance was measured at 450 nm using a Bio-Rad microplate reader. The cytokine concentrations were quantified on the basis of a linear dose−response standard curve.

The levels of antigen-specific IgE, IgG1, and IgG2a antibodies in the serum were determined by the ELISA method with biotin-conjugated rat antimouse IgE, IgG1, and IgG2a monoclonal antibodies (BD Biosciences, San Jose, CA). Disrupted cell suspension (200 μL) from GM-LAB or non-GM-LAB or 10 μg of OVA in PBS at pH 7.2 was coated on microtiter plates overnight at 4 °C. After being washed with PBST twice, the plates were blocked with 200 μL of 1% BSA in PBS for 1 h, and the washing step was repeated three times. Next, the plates were incubated with the diluted serum samples and washed with PBST before adding the biotinylated detection antibodies including IgE, IgG1, or IgG2a. The serum dilutions used were 1:25,000, 1:250, and 1:10 for IgG1, IgG2a, and IgE, respectively. Finally, the plates were incubated with streptavidin−HRP conjugate and tetramethylbenzidine substrate sequentially. Reaction was stopped by adding sulfuric acid, and the optical density was measured at 450 nm. The specific IgG1, IgG2a, and IgE titers were expressed as ELISA unit. The ELISA unit is calculated as the absorbance of sample subtracted by the blank ($A_{sample} - A_{blank}$) divided by the absorbance of positive control subtracted by the blank ($A_{positive} - A_{blank}$).[22] The absorbance of the positive control represented the levels of IgG1, IgG2a, and IgE in serum from OVA/CFA-immunized BALB/c mice.

Statistical Analysis. The experimental data were subjected to analysis of variance for a completely random design using the Statistical Analysis System (2008, SAS/STAT user's guide ver.9.1.3; SAS Institute, Inc., Cary, NC). Values are expressed as mean ± SD from seven animals. Duncan's multiple range test was used to determine the significant difference among means at a level of 0.01.

RESULTS

Pepsin Digestibility and Resistance. Resistance to pepsin digestion has been found in many food allergens. If a novel protein can resist digestion by pepsin, it may have allergenic potential.[6,23,24] We had introduced *apr*N from *B. subtilis* natto into *L. lactis* NZ9000, resulting in the GM *L. lactis* NZ9000 harboring the shuttle vector pNZPNK, which successfully produced NK. However, *L. lactis* NZ9000/pNZPNK was unable to secrete the NK out of cells. The cells of *L. lactis* NZ9000 and *L. lactis* NZ9000/pNZPNK were disrupted, and the total proteins (soluble and insoluble fractions) were evaluated. We used the SGF method to imitate the gastrointestinal tract of mammals. These proteins were digested with SGF for 0, 0.5, 2, 5, 10, 20, 30, and 60 min, and the reaction was stopped by heating. Tricine-SDS-PAGE analysis showed that NK produced by *L. lactis* NZ9000/pNZPNK existed in the insoluble fraction of cell lysate. STI was resistant to SGF digestion and remained stable after 60 min, but BSA was fully degraded after 5 min (Figure 3A). The partial degradation of cell debris by NK in *L. lactis* NZ9000/pNZPNK was observed at 30 s. After digestion for 5 min, the majority of proteins were digested into small fractions (Figure 3B). Western blotting analysis with NK-specific polyclonal antibody detected a protein fraction of 45 kDa, and the same fraction was digested after 5 min (Figure 3C). These results suggest that NK produced by GM *L. lactis* NZ9000/pNZPNK induced with nisin may not be an allergen. Further, the in vitro pepsin digestion assay may be an important means to assess the potential allergenicity of GMMs.

Clinical Observation and Relative Organ Weight. During the experimental period, all mice survived and showed no adverse effects such as enteritis, dermatitis, or infection. Furthermore, the body weights did not significantly differ among groups as evidenced by the body weights ranging from initial weights of 19.3 ± 0.9 g to final weights of 22.7 ± 2.0 g. After the mice were sacrificed, the hearts, livers, spleens, and kidneys were collected and weighed. The relative organ weights were similar among groups (Table 1).

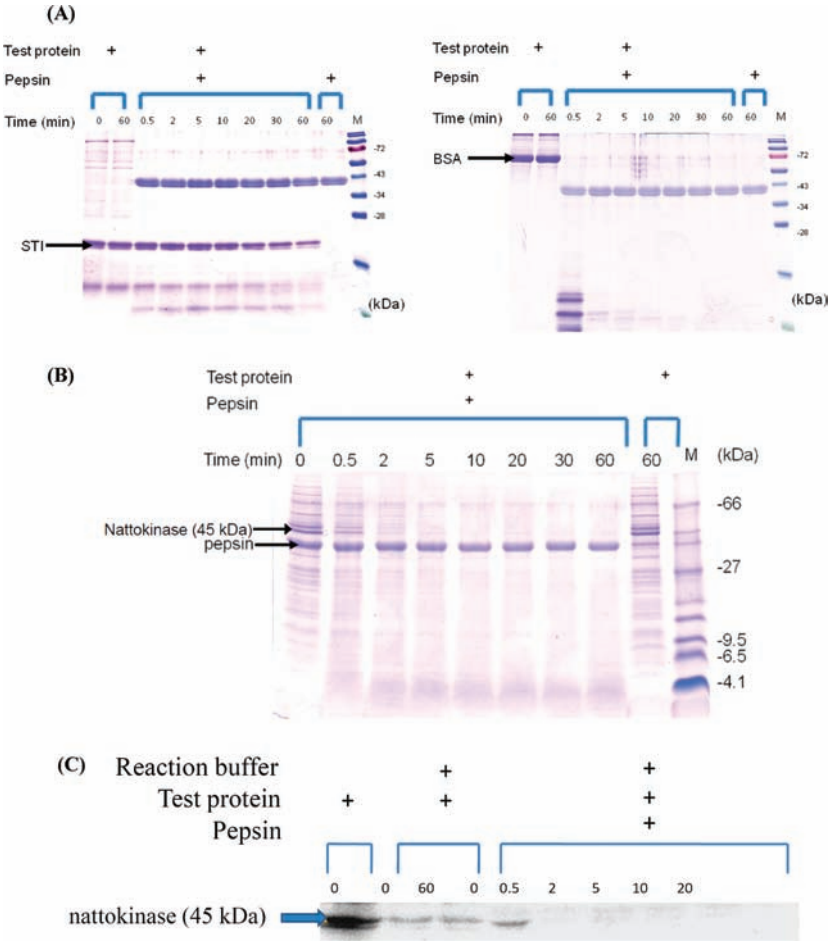

Figure 3. Pepsin digestibility of nattokinase from *L. lactis* NZ9000/pNZPNK. (A) SDS-PAGE analysis of pepsin-digested STI and BSA; (B) Tricine-SDS-PAGE of cell debris with SGF digestion for 0, 0.5, 2, 5, 10, 20, 30, and 60 min; and (C) Western blotting analysis of Tricine-SDS-PAGE gel using NK-specific polyclonal antibody to detect its protein fraction at 45 kDa.

Table 1. Body Weight and Relative Organ Weight of BALB/c Female Mice Orally Administrated with *L. lactis* NZ9000/pNZPNK and *L. lactis* NZ9000

	body weight (g)		relative organ weight[b] (%)			
treatment[a]	initial	final	spleen	liver	heart	kidney
CON	19.63 ± 1.30	20.77 ± 1.23	0.33 ± 0.07	3.97 ± 0.22	0.50 ± 0.03	1.16 ± 0.05
NZL	19.96 ± 0.57	21.37 ± 0.60	0.40 ± 0.07	4.08 ± 0.31	0.48 ± 0.04	1.18 ± 0.05
NZM	20.49 ± 2.15	21.54 ± 2.77	0.45 ± 0.21	4.49 ± 0.40	0.51 ± 0.08	1.14 ± 0.12
NZH	19.93 ± 1.91	20.89 ± 1.91	0.33 ± 0.04	4.29 ± 0.30	0.48 ± 0.03	1.13 ± 0.09
PNL	21.36 ± 2.00	22.71 ± 2.04	0.30 ± 0.08	4.28 ± 0.42	0.48 ± 0.04	1.18 ± 0.04
PNM	19.97 ± 1.52	21.39 ± 1.22	0.32 ± 0.09	4.40 ± 0.29	0.50 ± 0.02	1.20 ± 0.08
PNH	20.22 ± 1.93	21.90 ± 1.50	0.39 ± 0.17	4.19 ± 1.73	0.51 ± 0.06	1.25 ± 0.12
OVA	19.30 ± 0.92	20.36 ± 0.75	0.38 ± 0.19	4.14 ± 0.11	0.50 ± 0.02	1.17 ± 0.08

[a] CON, PBS group; NZL, low dosage of *L. lactis* NZ9000; NZM, moderate dosage of *L. lactis* NZ9000; NZH, high dosage of *L. lactis* NZ9000; PNL, low dosage of *L. lactis* NZ9000/pNZPNK; PNM, moderate dosage of *L. lactis* NZ9000/pNZPNK; PNH, high dosage of *L. lactis* NZ9000/pNZPNK; and OVA, ovalbumin group. Each value is expressed as mean ± SD (n = 7). [b] Relative organ weight = (organ weight/body weight) × 100%.

Effects of Lactococci on Splenocytes Proliferation in Mice. The effects of *L. lactis* NZ9000/pNZPNK (PN) or *L. lactis* NZ9000 (NZ) on splenocyte proliferation in BALB/c mice are shown in Figure 4. Administration of OVA decreased the proliferation of splenocytes stimulated by mitogen as compared to that of the normal control group. The results of the *L. lactis* NZ9000/pNZPNK group were similar to those of the OVA group. Feeding *L. lactis* NZ9000 at a high dosage with Con A stimulation induced a small increase in proliferation.

Effects of Lactococci on Cytokine Production. To evaluate the potential allergenicity of *L. lactis* NZ9000/pNZPNK or *L. lactis* NZ9000 in the gastrointestinal tract of BALB/c mice,

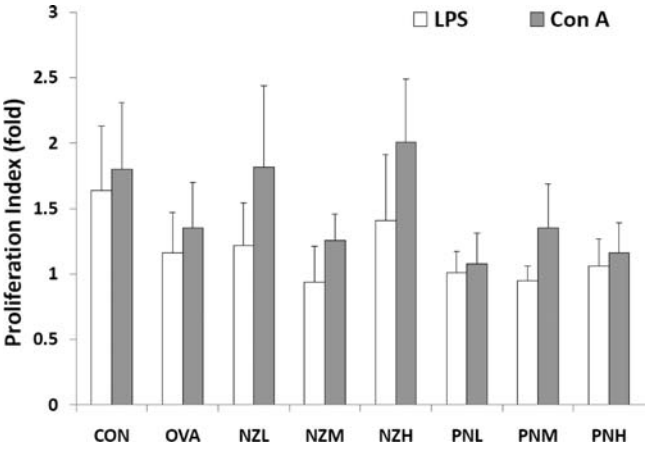

Figure 4. Proliferation indexes of splenocytes from BALB/c female mice orally administered with *L. lactis* NZ9000/pNZPNK and *L. lactis* NZ9000. Mice were fed with various dosages of lactococci for 42 days. Proliferation index (%) = OD of mitogen-stimulated culture/OD of nonstimulated culture. Means with asterisk were significantly different from the control group ($P < 0.01$). CON, PBS group; NZL, low dosage of *L. lactis* NZ9000; NZM, moderate dosage of *L. lactis* NZ9000; NZH, high dosage of *L. lactis* NZ9000; PNL, low dosage of *L. lactis* NZ9000/pNZPNK; PNM, moderate dosage of *L. lactis* NZ9000/pNZPNK; PNH, high dosage of *L. lactis* NZ9000/pNZPNK; OVA, ovalbumin group; LPS, lipopolysaccharide; Con A, concanavalin A.

the murine splenocytes were cultured in RPMI medium. The amounts of Th1 (IFN-γ and IL-12) and Th2 (IL-4 and IL-10) cytokines in the immune response were investigated, and the results are shown in Figure 5. As shown in Figure 5A, oral administration of the low dose of *L. lactis* NZ9000 significantly increased the secretion of IFN-γ (585.0 $\pm$ 181.3 pg/mL, $P <$ 0.01), while feeding with the high dose of *L. lactis* NZ9000/pNZPNK decreased the amount of IFN-γ as compared to that of the control group.

It also shown that secretion of IL-12 in *L. lactis* NZ9000/pNZPNK groups was similar to that of the matched control (Figure 5B). The amount of IL-12 in the high dose of *L. lactis* NZ9000 was slightly decreased. The secretions of IL-4 by feeding with moderate and high doses of *L. lactis* NZ9000 were 26.7 $\pm$ 10.8 and 28.5 $\pm$ 7.1 pg/mL (Figure 5C), respectively. The amounts of IL-10 by feeding with all doses of *L. lactis* NZ9000/pNZPNK were significantly decreased to 27.6−280.2 pg/mL as compared to that of the control group (802.5 pg/mL, Figure 5D). The balance between Th1 and Th2 cells was important in assessing immunoregulation, and it could be derived from the IFN-γ/IL-4 ratio (Figure 5E). The IFN-γ/IL-4 ratio of the high dose *L. lactis* NZ9000 group (61.2 $\pm$ 17.1) markedly increased as compared to the control group (24.5 $\pm$ 12.7) and improved the Th1/Th2 balance toward Th1 dominance.

Effects of Lactococci on Specific Serum Screening of IgG1, IgG2a, and IgE. The serum levels of LAB-total protein or OVA-specific antibodies (IgG1, IgG2a, and IgE) are shown in Figure 6. Blood was collected at the beginning of the test and biweekly thereafter. The results indicated that the OVA-specific IgG1 and IgE levels (1.3−1.6 and 0.9−1.0 EU, respectively) markedly increased at weeks 2 and 4 as compared to those of the other groups (Figure 6A and B). The levels of LAB-specific IgG2a in the groups fed with moderate and high dosages of *L. lactis* NZ9000 and all dosages of *L. lactis* NZ9000/pNZPNK (2.9−3.8 and 2.5−3.1 EU, respectively) were significantly greater than those in the PBS or OVA group at week 2 (Figure 6C, $P < 0.01$), but no significant difference was found at the end of the treatment period.

DISCUSSION

Recently, many researchers have focused on the strain improvement of lactococci by using gene technology.[25,26] To eliminate the potential adverse affects of the gene products, especially in toxic or allergenic proteins, the risk should be assessed, and appropriate strategies for risk management may be required. In this study, we used methods including screening of specific serum immunoglobulin and intestinal pepsin resistance to assess the allergenicity of the gene product and of the whole bacteria.

The results of the in vitro pepsin digestibility and stability assays of NK in our study are shown in Figure 2. The protein NK has been safely used for many centuries. It is broken down by pepsin or other digestive enzymes in the gastrointestinal tract. However, because stability is not the only feature shared by all known allergens, we cannot depend on indigestibility as the sole determining factor in the risk assessment of novel proteins.[7]

For spleen cells stimulated with or without Con A and LPS, this is known by stimulating the T- and B-cells proliferation, respectively. The secretion of the Th2 cytokines IL-4 and IL-10 in mice fed with *L. lactis* NZ9000/pNZPNK or *L. lactis* NZ9000 was significantly lower than that in the OVA group (Figure 5). It shows that oral administration of *L. lactis* NZ9000/pNZPNK or *L. lactis* NZ9000 could suppress the Th2 response and act as a key factor to inhibit the overproduction of IgE. The balance between Th1 and Th2 has an important role in the immunoregulation of various diseases.[27]

The amount of IFN-γ was not expected to increase in the *L. lactis* NZ9000/pNZPNK groups. Therefore, oral administration of *L. lactis* NZ9000/pNZPNK did not alter the Th1/Th2 balance as compared to the control group. Regulation of the Th1/Th2 balance based on the IFN-γ/IL-4 ratio indicated that feeding with a higher dosage of *L. lactis* NZ9000 produced significantly higher ratios than the other groups, which might be explained by their good colonization or transiently resident functions in the gastrointestinal tract[20,28−30] and immunomodulatory activity by the enhancement of the Th1-type immune response.[31−33]

It is required that novel food proteins be assessed for their allergenic potential before introducing them to the food market. All guidance documents are based on an array of tests proposed by the FAO/WHO.[2] The animal model is not a necessary test in the weight-of-evidence approach from the FAO/WHO.[2] Nevertheless, this model may actually identify and distinguish the allergenic properties of truly novel proteins from confirmed allergenic proteins.[9,10,34] An ideal animal model must be specified by possessing responses and relative activities similar to those in humans, eliciting a response from exposure routs, not requiring adjuvant, and relatively saving time and money.[35] Examples of murine models of food allergy that have been reported involve the following strains: BALB/c, DBA/2, C3H/HeJ, A/J, BDF-1, and the C57BL/6. On the other hand, the Brown Norway (BN) strain is considered the most consistent and highest responder for rats.[12] To compare the results between our research and others, the immune protocol with intraperitoneal injection route was similar, and the specific IgE was determined as well. In our study, the number of mice in each group ($n = 8$) was higher than

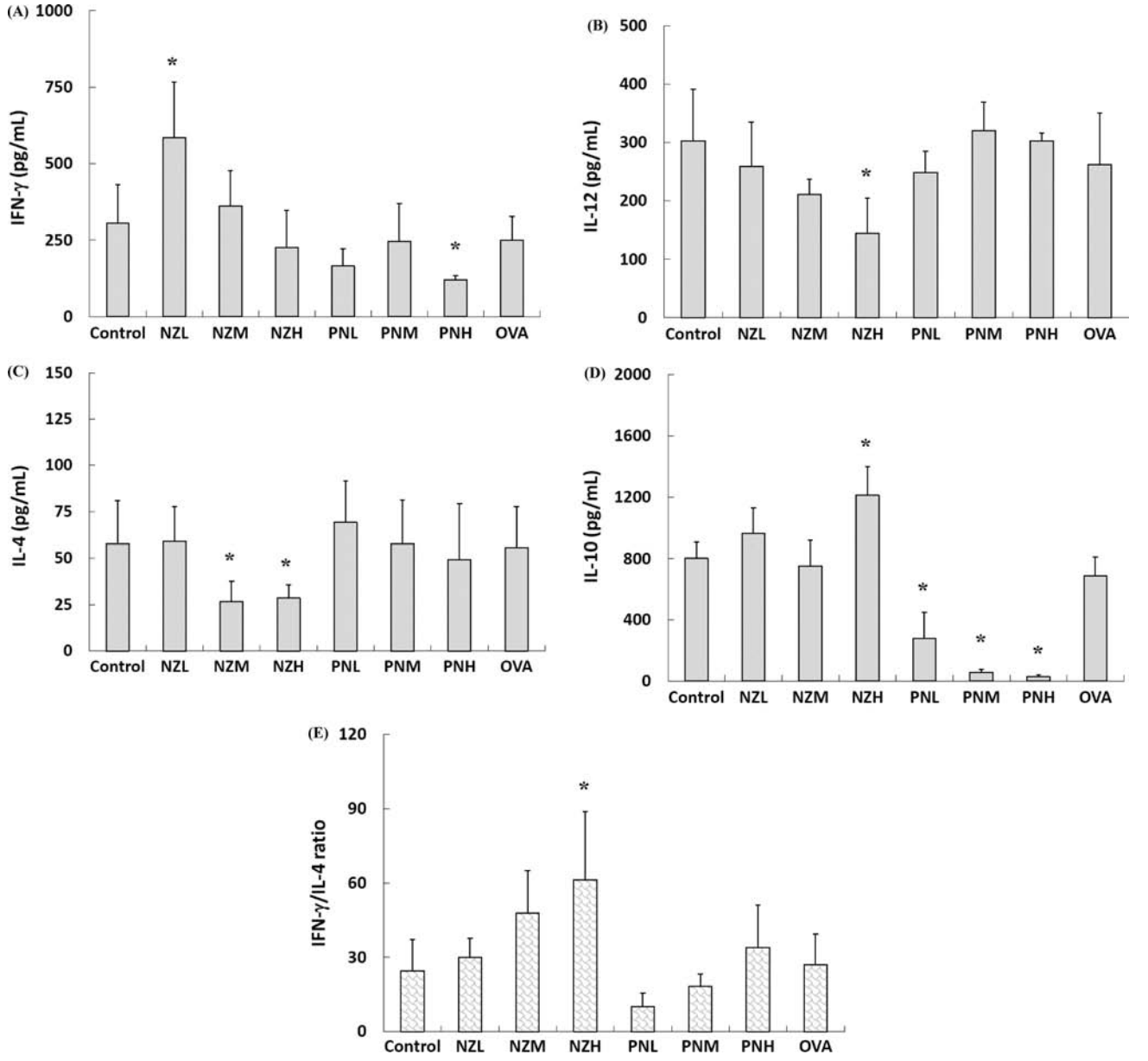

Figure 5. Cytokine content and IFN-γ/IL-4 ratio of splenocytes from BALB/c female mice orally administered with *L. lactis* NZ9000/pNZPNK and *L. lactis* NZ9000. (A) IFN-γ; (B) IL-12; (C) IL-4; (D) IL-10; (E) Th1/Th2 balance as the ratio of IFN-γ to IL-4 concentration. Means with asterisk were significantly different from the control group ($P < 0.01$). CON, PBS group; NZL, low dosage of *L. lactis* NZ9000; NZM, moderate dosage of *L. lactis* NZ9000; NZH, high dosage of *L. lactis* NZ9000; PNL, low dosage of *L. lactis* NZ9000/pNZPNK; PNM, moderate dosage of *L. lactis* NZ9000/pNZPNK; PNH, high dosage of *L. lactis* NZ9000/pNZPNK; OVA, ovalbumin group; IL, interleukin.

in other research (n = 3 or 4). We used the specific serum antibody screening to investigate the potential protein binding to primary immunogen in the murine model. This method was based on the assessment of induced antibody responses and the number of responders in the test groups.[8,36,37] It also needs more efforts or noninvasive methods to evaluate the allergenicity in animals or human models. Even in human oral immunotherapy trials, it has to be mentioned that the laboratory should use the whole foods rather than proteins as test materials. In our study, the results showed that BALB/c mice orally administered with OVA had higher contents of specific IgG1 and IgE than those treated with *L. lactis* NZ9000/pNZPNK or *L. lactis* NZ9000. The serum IgG2a contents of lactococci increased dose-dependently at week 2, and it would be an effector between Th1 and Th2 cells. In addition to various effector pathways by virtue of their lymphokine production (such as high INF-γ and low IL-4), so Th1 cells switch off Th2 cells. It may show that the Th1 relative antibody markedly increased at early stage and induced immune reaction toward the Th1 pathway.

In conclusion, it can be said that oral administration of *L. lactis* NZ9000/pNZPNK does not increase the proliferation of type 2 helper T cells or the secretion of cytokines in the spleen, and thus the strain has a low risk of inducing the production of serum-specific antibodies and allergenicity in the BALB/c mouse model. Accordingly, this GM LAB may not only serve as a probiotic but also be a safe dietary supplement in human health.

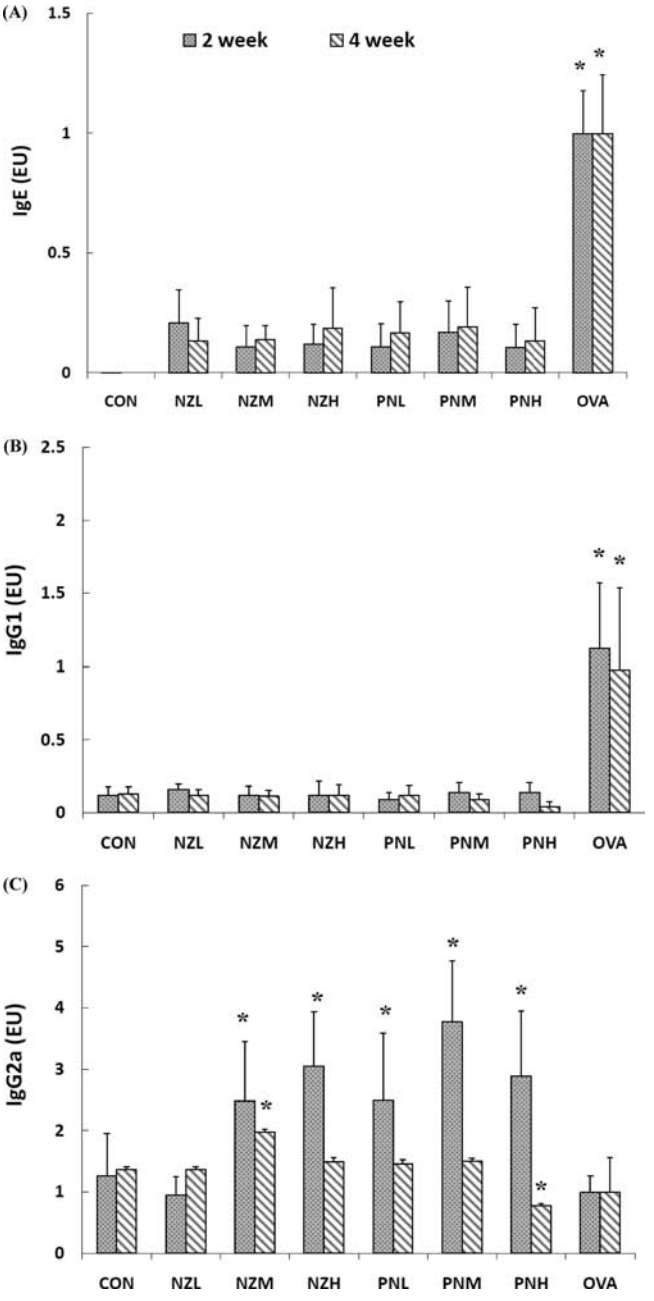

Figure 6. Changes in specific IgE (A), IgG1 (B), and IgG2a (C) antibodies titers in BALB/c female mice orally administered with *L. lactis* NZ9000/pNZPNK and *L. lactis* NZ9000. Means with asterisk were significantly different from the control group ($P < 0.01$). CON, PBS group; NZL, low dosage of *L. lactis* NZ9000; NZM, moderate dosage of *L. lactis* NZ9000; NZH, high dosage of *L. lactis* NZ9000; PNL, low dosage of *L. lactis* NZ9000/pNZPNK; PNM, moderate dosage of *L. lactis* NZ9000/pNZPNK; PNH, high dosage of *L. lactis* NZ9000/pNZPNK; OVA, ovalbumin group; IL, interleukin; LPS, lipopolysaccharide; and Con A, concanavalin A. EU, ELISA unit [$(A_{sample} - A_{blank})/(A_{positive} - A_{blank})$].

ABBREVIATIONS USED

BSA, bovine serum albumin; Con A, concanavalin A; GM, genetically modified; GMM, genetically modified microorganism; HRP, horseradish peroxidase; LAB, lactic acid bacteria; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NK, nattokinase; OVA, ovalbumin; PBS, phosphate buffered saline; PBST, PBS containing 0.05% Tween-20; SGF, stimulated gastric fluid; STI, soybean trypsin inhibitor.

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