In vivo dose response and in vitro mechanistic analysis of enhanced immunoglobulin A production by Lactobacillus plantarum AYA

Yosuke KIKUCHI1*, Hikaru YOSHIDA2, Tasuku OGITA2, Kimiko OKITA3, Shin-ichi FUKUDOME1, Takuya SUZUKI2 and Soichi TANABE2

1Research Center for Basic Science, Research, and Development, Quality Assurance Division, Nisshin Seifun Group Inc., 5-3-1 Tsurugaoka, Fujimino, Saitama 356-8511, Japan
2Graduate School of Biosphere Science, Hiroshima University, 1-4-4 Kagamiyama, Higashihiroshima, Hiroshima 739-8528, Japan
3Yeast Function Development Unit, Oriental Yeast Co., Ltd., 3-6-10 Azusawa, Itabashi, Tokyo 174-8505, Japan

Secretory immunoglobulin A (IgA) mediates the mucosal immune system, which provides the first line of defense against inhaled and ingested pathogenic bacteria and viruses. Lactobacillus plantarum AYA increases the IgA level of Peyer’s patch (PP) cells, but the recommended amount of consumption and the mechanism of action remains unclear. Better understanding of these is essential to development of L. plantarum AYA for use in functional foods. Therefore, we investigated the dose-response effect (in vivo) and mechanism (in vitro) of IgA enhancement induced by L. plantarum AYA. In the small intestine of the mice fed a diet containing 0.03% or 0.3% of L. plantarum AYA powder for 4 weeks, the IgA levels were significantly increased. Thus, it is suggested that the recommended amount of consumption of L. plantarum AYA is about 0.72 mg per day. In addition, the bacterial cell wall fraction significantly enhanced the IgA production level of murine PP cells in the in vitro assay. The ability of whole cells and the cell wall fraction to enhance IgA levels was significantly inhibited by an anti-Toll-like receptor-2 (TLR-2) antibody, which suggests that the cell wall fraction of L. plantarum AYA increases the IgA level via TLR-2. These findings indicate that L. plantarum AYA is a potential functional food source that maintains mucosal immunity.

Key words: Lactobacillus plantarum AYA, Peyer’s patch, Toll-like receptor 2, immunoglobulin A, cell wall, probiotics

INTRODUCTION

The mucosal immune system defends the body from foreign organisms and substances, such as pathogens and their secreted products, viruses and food allergens and is intimately involved in homeostasis [1, 2]. In particular, mucosal immunity is critically important for protecting the mucous membrane of the gut because its large surface area is exposed to the external environment. During evolution, humans developed gut-associated lymphoid tissue, an indispensable immune system that comprises Peyer’s patches (PPs), the lamina propria of the mucous membrane, intraepithelial lymphocytes and mesenteric lymph nodes [3]. This system is centered on immunoglobulin A (IgA) that is secreted from the intestinal mucosa to prevent the pathogenic viruses and bacteria from infecting the epithelial cells and to neutralize the toxins produced by the bacteria [4–6]. The PPs, which are distributed around the luminal surface of the small intestine, contain cells required to generate an immune response, such as dendritic cells, macrophages, T cells, and B cells [3]. Therefore, the immune response leading to antibody production is likely initiated by the PPs [5].

Oral administration of a diet containing 5% Lactobacillus plantarum AYA (FERM P-21106) significantly enhances IgA levels in the small intestine of mice [7, 8]. L. plantarum AYA enhances interleukin-6 (IL-6) production by the dendritic cells in the PPs, leading to an increased IgA level by inducing IgA+ B cells to differentiate into plasma cells that secrete IgA [7]. However, it is not yet clear whether L. plantarum AYA could be effective as a component of functional foods. It is necessary to determine the recommended amount of consumption for development as a functional...
food because the minimum amount of *L. plantarum* AYA required is unknown. Also, mechanistic analysis of the enhanced IgA production caused by *L. plantarum* AYA is strongly required.

Lactobacilli have been shown to enhance IgA secretion from the intestinal mucosa [9, 10]. For example, *L. rhamnosus* GG [11] and *L. johnsonii* NCC533 (La1) [12] enhanced IgA secretion in an animal experiment, and *L. gasseri* SBT2055 enhanced the IgA production levels of PP cell cultures in another animal experiment [13]. In general, the substances in lactobacilli that modulate the immune response include peptidoglycans [14–16], lipoproteins [15, 16], lipoteichoic acid [15–17], and DNA [15, 16, 18, 19]. When these substances bind to Toll-like receptors (TLRs) expressed by PP cells, they induce production of various cytokines that activate immune responses [15, 16, 20–22].

The aim of the present study was to investigate whether *L. plantarum* AYA could be useful as a component of functional foods. In this study, we examined the in vivo dose-response effect and in vitro mechanism of IgA enhancement induced by *L. plantarum* AYA.

**MATERIALS AND METHODS**

**Mice**

Female BALB/c mice, aged 6–9 weeks, were obtained from Japan SLC (Shizuoka, Japan) and Charles River Laboratories Japan (Kanagawa, Japan). All mice were housed under specific pathogen free conditions and were provided AIN-93G diet (Oriental Yeast Co., Ltd., Tokyo, Japan) and water *ad libitum* under a 12-hr light-dark cycle (light, 08:00–20:00; dark, 20:00–08:00). The Animal Care and Use Committee of the Nisshin Seifun Group and Hiroshima University approved the animal experiments.

**L. plantarum AYA and culture conditions**

*L. plantarum* AYA was obtained from the Oriental Yeast Co., Ltd., cultured in sterile GYP broth at pH 6.8, and incubated at 30°C for 24 hr. The GYP broth contained 1% glucose, 1% yeast extract, 0.5% Bacto Peptone, 0.2% sodium acetate (CH₃COONa · 3H₂O), 20 ppm magnesium sulfate heptahydrate (MgSO₄ · 7H₂O), 1 ppm manganese sulfate (MnSO₄), 1 ppm ferrous sulfate heptahydrate (FeSO₄ · 7H₂O), 1 ppm sodium chloride (NaCl) and 2.5 ppm Tween 80. The cells were harvested by centrifugation at 5,000 × g for 10 min and then washed three times with sterile saline. The washed cells were sterilized in an autoclave at 121°C for 30 min and then lyophilized. Therefore, the *L. plantarum* AYA preparation used in this study was a killed bacteria powder.

**Preparation of the cell wall fraction of *L. plantarum* AYA**

In brief, to prepare the cell wall fraction, 0.5 g of powdered *L. plantarum* AYA was suspended in 20 ml of 0.3% sodium dodecyl sulfate solution (Wako Pure Chemical Industries, Osaka, Japan) [23]. The suspension was centrifuged at 1,400 × g for 10 min, and the supernatant was discarded. The precipitate (pellet) was rinsed three times with sterilized water and once with acetone. The precipitate was then suspended in 10 ml of methanol, it was centrifuged at 1,400 × g for 10 min, and the supernatant was then discarded. The precipitate was further resuspended in 20 ml of Benzonase solution (Sigma-Aldrich, St. Louis, MO, USA) containing 50 mM Tris-HCl buffer (pH 8.0) and 10 U/ml of Benzonase, and incubated at 37°C for 20 hr. The mixture was then centrifuged at 1,400 × g for 10 min, and the supernatant was discarded. After the precipitate was resuspended in 10 ml of methanol, it was centrifuged at 1,400 × g for 10 min, and the supernatant was then discarded. The precipitate was further resuspended in 20 ml of Pronase solution (Wako Pure Chemical Industries) containing 50 mM Tris-HCl buffer (pH 6.8) and 1 mg/ml of Pronase, and incubated at 37°C for 20 hr. After the suspension was centrifuged at 1,400 × g for 10 min, the precipitate was resuspended in 20 ml of Pronase solution and incubated at 37°C for 72 hr. The suspension was centrifuged at 1,400 × g for 10 min, and the precipitate was rinsed three times with sterilized water and lyophilized. The product was used as the cell wall fraction.

**Preparation of the DNA fraction of *L. plantarum* AYA**

To prepare the DNA fraction, 0.1 g of powdered *L. plantarum* AYA was suspended in 1 ml of TRIzol (Life Technologies, Carlsbad, CA, USA). The suspension was vigorously shaken for 10 sec and incubated at room temperature for 5 min. Then, 200 μl of chloroform was added, and the solution was shaken vigorously for 15 sec before incubation at room temperature for 5 min. After the suspension was centrifuged at 1,400 × g for 10 min, the aqueous (RNA) phase was removed. Then 300 μl of 99.5% ethanol was added to the interphase and the organic phase, and the solution was shaken vigorously for 10 sec and incubated at room temperature for 3 min. After the suspension was centrifuged at 2,000 × g for 5 min, the precipitate (pellet) was rinsed with 1 mL of 10% ethanol containing 0.1 M sodium acetate at room temperature for 30 min. Next, the suspension was centrifuged at 2,000 × g for 5 min at 4°C, and the supernatant was discarded. The precipitate was then rinsed with 1 ml of 75% ethanol at room temperature for 20 min. The suspension was subsequently centrifuged at 2,000 × g for 5 min, and...
the supernatant was discarded. The product was used as the DNA fraction. DNA was eluted using 300 µl of 8 mM NaOH. After adjusting the pH to 7.0 using 1 M HEPES, DNA was quantitated with a NanoDrop ND-100 (Labtech International Ltd., Uckfield, UK).

Mouse experiments

The mice were divided into three groups (all n=6) with similar average body weights (18.8 g). Each group was fed either a normal diet (AIN93G) or a diet containing 0.03% or 0.3% of *L. plantarum* AYA powder *ad libitum* for 4 weeks. The mice were then sacrificed by cervical dislocation, and the small intestine was removed, divided into upper and lower segments and flushed with 1 ml of phosphate-buffered saline (PBS) containing cOmplete EDTA-free protease inhibitor cocktail (Roche Diagnostics, Rotkreuz, Switzerland). One cOmplete EDTA-free protease inhibitor cocktail tablet was used for 50 ml of PBS. The supernatant was collected after the washed fluid was centrifuged at 9,200 × g at 4°C for 30 min.

Isolation of PP cells

PP cells were collected from the mucosa of the small intestine and incubated in RPMI 1640 medium (Sigma-Aldrich) with 10% fetal calf serum (FCS; Sigma-Aldrich) and 0.2 U of collagenase type I (1 mg/ml, Sigma-Aldrich) on a magnetic stirrer at 37°C for 60 min. The cell suspensions were then passed through a 70 µm nylon mesh cell strainer (BD Biosciences, San Jose, CA, USA), centrifuged at 300 × g at 4°C for 5 min, washed with PBS, shaken vigorously for 10 sec and again centrifuged at 300 × g at 4°C for 5 min. After two additional washes, the supernatant was aspirated completely. This procedure yielded the population of PP cells.

Analysis of the IgA production level of PP cells

Cells were cultured in RPMI 1640 medium containing 10% FCS, 2 mM of l-glutamine, 100 U/ml of penicillin and 100 µg/ml of streptomycin (designated complete medium) in a humidified atmosphere. The PP cells were plated at 10⁶ cells per well. First, the ability of the cell wall fraction of *L. plantarum* AYA to increase the IgA production level was evaluated. The PP cells were cultured with either 100 or 500 µg/ml of the *L. plantarum* AYA powder or 100 or 500 µg/ml of cell wall fraction. Supernatants were collected after 72 hr, and IgA concentrations were measured using ELISA.

Quantification of IgA

IgA concentrations in small intestine fluid or cell culture supernatants were determined using a sandwich ELISA kit (Mouse IgA ELISA Quantitation Set; Bethyl Laboratories, Montgomery, TX, USA). The kit was used according to the manufacturer’s instructions. In brief, 96-well immunoplates were coated with antibodies. After washing and blocking the immunoplates, the samples and standards were added, followed by incubation. After washing, a horseradish peroxidase-labeled monoclonal antibody was added, and the plates were incubated at room temperature for 1 hr. The plates were washed again and incubated with the substrate 3,3′,5,5′-tetramethylbenzidine (Moss Inc., Elk Grove Village, IL, USA). A stop solution was added, and absorbance was measured at 450 nm.

Statistical analysis

We compared the variances in data among the groups. If the variances were equal, we used parametric evaluation to identify statistically significant differences between the groups using the Tukey test. If the variances were unequal, we used a nonparametric evaluation such as the Steel-Dwass test. Differences with p<0.05 were considered significant.

RESULTS AND DISCUSSION

IgA levels in the small intestine of mice fed a diet containing killed, powdered *L. plantarum* AYA

Various lactobacilli strains [11–14], as well as a powdered form of *L. plantarum* AYA [7], that enhance IgA secretion have been described. The objectives of this study were to determine the recommended amount of consumption and the mechanism underlying the enhancement of IgA by *L. plantarum* AYA.

Oral administration of a diet containing 5% (by weight) *L. plantarum* AYA powder *ad libitum* for 28 days significantly enhanced the IgA levels in the small intestine of the mice [7]. However, the minimum amount of *L. plantarum* AYA required is unknown. In the present study, the IgA levels were significantly enhanced in the small intestine of the mice fed a diet containing 0.03% or 0.3% of *L. plantarum* AYA powder in a dose-dependent manner (Fig. 1). The mean ± standard deviation (SD) body weights were 21.4 ± 1.4 g, 21.7 ± 1.3 g and 21.5 ±
1.6 g in the control, 0.03% and 0.3% groups, respectively. The average food consumptions were 2.38 g/day, 2.41 g/day and 2.39 g/day in the control, 0.03% and 0.3% groups, respectively. There was no significant difference in body weight or food consumption among the three groups. Using the average food consumption, we calculated the consumption of L. plantarum AYA. The mice consumed approximately 0.72 mg and 7.2 mg of L. plantarum AYA per day in the 0.03% and 0.3% groups, respectively. Therefore, a diet containing 0.72 mg per day (equivalent to 8.6×10^8 colony forming units) of killed, powdered L. plantarum AYA was sufficient to increase IgA levels in the intestine. It is suggested that the recommended amount of consumption of L. plantarum AYA is 0.72 mg per day in mice.

**IgA production level of PP cells treated with the cell wall fraction and DNA of L. plantarum AYA**

Lactobacilli components with immune modulatory activity include peptidoglycans [14–16] and lipoteichoic acid [15–17], which are present in the cell wall, and DNA [15, 16, 18, 19]. The data presented in Fig. 2 shows that the cell wall fraction of L. plantarum AYA enhanced the IgA production level of PP cells. Therefore, at least one of the active compounds that enhance the IgA production level was present in the cell wall of L. plantarum AYA.

However, the amount of IgA secreted by the PP cells exposed to the cell wall fraction was significantly less than that secreted in response to unfractionated L. plantarum AYA. The IgA production level of the PP cells was not significantly enhanced by 100 µg/ml of the cell wall fraction, but 500 µg/ml did significantly enhance the IgA production level (p<0.05). However, both 100 µg/ml and 500 µg/ml of unfractionated L. plantarum AYA significantly enhanced the IgA production level (p<0.05; Fig. 2). There is approximately 30 µg of the cell wall fraction in 100 µg of unfractionated L. plantarum AYA. So, the concentration of the cell wall fraction is 30 µg/ml in 100 µg/ml of unfractionated L. plantarum AYA. Therefore, unfractionated L. plantarum AYA increased the IgA production level to a greater extent than the cell wall fraction, which indicates that components other than those present in the cell wall fraction also enhance the IgA production level.
MECHANISM AND DOSE EFFECT OF IGA ENHANCEMENT BY L. PLANTARUM

It is known that TLR-9 agonist also stimulates IgA secretion in the intestine [24, 25]. Therefore, DNA fractions isolated from L. plantarum AYA were tested to determine whether they enhanced IgA secretion by the PP cell cultures. In the same in vitro assay, the DNA fraction (2 µg/ml) of L. plantarum AYA enhanced the IgA production level to 1.6 times that of the control. However, the involvement of DNA in enhancement the IgA production level by L. plantarum AYA is likely to be negligible, because an excessive amount of DNA was required for this activity.

An anti-TLR-2 antibody blocks the effect of the cell wall fraction on enhancement of the IgA production level

TLR-2 is a receptor for the peptidoglycan and lipoteichoic acid that are components of the cell wall of Gram-positive bacteria [20], and L. pentosus strain b240 enhances the IL-6 and IgA levels through TLR-2 [14]. Therefore, L. plantarum AYA might also enhance the IgA level through TLR-2. The effect of an anti-TLR-2 antibody on the production of IgA by cultures of PP cells was evaluated. The ability of the cell wall fraction to enhance IgA secretion was almost completely suppressed by addition of the anti-TLR-2 antibody, although the antibody had no effect on untreated cells (Fig. 3).

In conclusion, IgA production mediates the mucosal immune system, which provides the first line of defense against inhaled and ingested pathogenic microbacteria and viruses. From the in vivo experiment, it was suggested that a diet containing 0.72 mg per day of L. plantarum AYA was sufficient to increase the IgA levels in the intestine. In the in vitro experiment using murine PP cells, IgA enhancement was induced by the cell wall fraction of L. plantarum AYA, and it was mediated by TLR-2. These findings may lead to the development of new functional foods based on the addition of L. plantarum AYA.

ACKNOWLEDGEMENT

The authors wish to thank Dr. Hachimura (Tokyo University) for his technical support and valuable discussion.

REFERENCES

1. Holmgren J, Czerkinsky C. 2005. Mucosal immunity and vaccines. Nat Med 11 Suppl: S45–S53. [Medline] [CrossRef]
2. Brandtzaeg P, Baekkevold ES, Farstad IN, Jahnsen FL, Johansen FE, Nilsen EM, Yamanaka T. 1999. Regional specialization in the mucosal immune system: what happens in the microcompartments? Immunol Today 20: 141–151. [Medline] [CrossRef]
3. Wershil BK, Furuta GT. 2008. Gastrointestinal mucosal immunity. J Allergy Clin Immunol 121 Suppl: S380–S383, quiz S415. [Medline] [CrossRef]
4. Cerutti A, Rescigno M. 2008. The biology of intestinal immunoglobulin A responses. Immunity 28: 740–750. [Medline] [CrossRef]
5. Fagarasan S, Honjo T. 2003. Intestinal IgA synthesis: regulation of front-line body defences. Nat Rev Immunol 3: 63–72. [Medline] [CrossRef]
6. Macpherson AJ, McCoy KD, Johansen FE, Brandtzaeg P. 2008. The immune geography of IgA induction and function. Mucosal Immunol 1: 11–22. [Medline] [CrossRef]
7. Kikuchi Y, Kunitoh-Asari A, Hayakawa K, Imai S, Kasuya K, Abe K, Adachi Y, Fukudome S, Takahashi Y, Hachimura S. 2014. Oral administration of Lactobacillus plantarum strain AYA enhances IgA secretion and provides survival protection against influenza virus infection in mice. PLoS ONE 9: e86416 http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0086416. [Medline] [CrossRef]
8. Kikuchi Y, Toyoda T, Ishijima T, Abe K, Nakai Y, Fukudome S. 2014. Oral administration of the AYA strain of Lactobacillus plantarum modulates expression of immunity-related genes in the murine Peyer’s patch: a DNA microarray analysis. Biosci Biotechnol Biochem 78: 1935–1938. [Medline] [CrossRef]
9. Blum S, Haller D, Pfeifer A, Schißfrin EJ. 2002. Probiotics and immune response. Clin Rev Allergy Immunol 22: 287–309. [Medline] [CrossRef]

10. Forchielli ML, Walker WA. 2005. The role of gut-associated lymphoid tissues and mucosal defence. Br J Nutr 93 Suppl 1: S41–S48. [Medline] [CrossRef]

11. He F, Morita H, Kubota A, Ouwehand AC, Hosoda M, Hiramatsu M, Kurisaki J. 2005. Effect of orally administered non-viable Lactobacillus cells on murine humoral immune responses. Microbiol Immunol 49: 993–997. [Medline] [CrossRef]

12. Inoue R, Nishio A, Fukushima Y, Ushida K. 2007. Oral treatment with probiotic Lactobacillus johnsonii NCC533 (La1) for a specific part of the weaning period prevents the development of atopic dermatitis induced after maturation in model mice, NC/Nga. Br J Dermatol 156: 499–509. [Medline] [CrossRef]

13. Sakai F, Hosoya T, Ono-Omachi A, Ubike K, Ogawa A, Moriya T, Kadooka Y, Shiozaki T, Nakagawa H, Nakayama Y, Miyazaki T. 2014. Lactobacillus gasseri SB2055 induces TGF-β expression in dendritic cells and activates TLR2 signal to produce IgA in the small intestine. PLoS ONE 9: e105370 http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0105370. [Medline] [CrossRef]

14. Kawai T, Akira S. 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 11: 373–384. [Medline] [CrossRef]

15. Mayer ML, Phillips CM, Stadnyk AW, Halperin SA, Lee SF. 2009. Synergistic BM-DC activation and immune induction by the oral vaccine vector Streptococcus gordonii and exogenous tumor necrosis factor. Mol Immunol 46: 1883–1891. [Medline] [CrossRef]

16. Mayer ML, Phillips CM, Townsend RA, Halperin SA, Lee SF. 2009. Differential activation of dendritic cells by Toll-like receptor agonists isolated from the Gram-positive vaccine vector Streptococcus gordonii. Scand J Immunol 69: 351–356. [Medline] [CrossRef]

17. Mohamadzadeh M, Pfeiler EA, Brown JB, Zadeh M, Gramarossa M, Managlia E, Bere P, Sarraj B, Khan MW, Pakanati KC, Ansari MJ, O’Flaherty S, Barrett T, Klaenhammer TR. 2011. Regulation of induced colonic inflammation by Lactobacillus acidophilus deficient in lipoteichoic acid. Proc Natl Acad Sci USA 108 Suppl 1: 4623–4630. [Medline] [CrossRef]

18. Kitazawa H, Watanabe H, Shimosato T, Kawai Y, Itoh T, Saito T. 2003. Immunostimulatory oligonucleotide, CpG-like motif exists in Lactobacillus delbrueckii ssp. bulgaricus NIAI B6. Int J Food Microbiol 85: 11–21. [Medline] [CrossRef]

19. Shimosato T, Kitazawa H, Katoh S, Tohno M, Iliev ID, Nagasawa C, Kimura T, Kawai Y, Saito T. 2005. Augmentation of T(H)-1 type response by immunoreactive AT oligonucleotide from lactic acid bacteria via Toll-like receptor 9 signaling. Biochem Biophys Res Commun 326: 782–787. [Medline] [CrossRef]