

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

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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<sup>+</sup> 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

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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 ( $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$ ), 20 ppm magnesium sulfate heptahydrate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ), 1 ppm manganese sulfate ( $\text{MnSO}_4$ ), 1 ppm ferrous sulfate heptahydrate ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ), 1 ppm sodium chloride (NaCl) and 2.5 ppm Tween 80. The cells were harvested by centrifugation at  $5,000 \times g$  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  $1,400 \times g$  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 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. The mixture was then centrifuged at  $1,400 \times g$  for 10 min, and the supernatant was discarded. After the precipitate was resuspended in 10 ml of methanol, it was centrifuged at  $1,400 \times g$  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 was incubated at 37°C for 20 hr. After the suspension was centrifuged at  $1,400 \times g$  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  $1,400 \times g$  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  $\mu\text{l}$  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  $1,400 \times g$  for 10 min, the aqueous (RNA) phase was removed. Then 300  $\mu\text{l}$  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  $2,000 \times g$  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  $2,000 \times g$  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  $2,000 \times g$  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  $9,200 \times g$  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  $300 \times g$  at 4°C for 5 min, washed with PBS, shaken vigorously for 10 sec and again centrifuged at  $300 \times g$  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  $10^6$  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.

The next part of the experiment was to determine whether TLR-2 mediated the IgA production level of PP cells treated with the *L. plantarum* AYA powder (500 µg/

ml) or cell wall fraction (500 µg/ml). The PP cells were co-incubated with or without a monoclonal anti-TLR-2 antibody (5 µg/ml). 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 [ $\pm$  standard deviation (SD)] body weights were  $21.4 \pm 1.4$  g,  $21.7 \pm 1.3$  g and  $21.5 \pm$

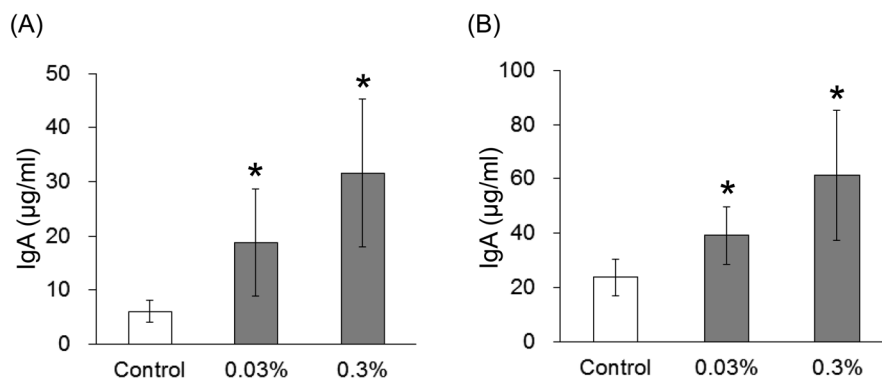


Fig. 1. IgA concentrations in the small intestine of the mice fed a diet containing *L. plantarum* AYA. BALB/c mice ( $n = 6$  for each group) were fed a control diet or a diet containing *L. plantarum* AYA 0.03% or 0.3% by weight for 28 days. IgA concentrations in fluids from the upper (A) and lower (B) small intestine were assayed using ELISA. The data represent the mean  $\pm$  standard deviation (SD) ( $n = 6$ ). \* $p < 0.05$  versus control (Steel–Dwass test).

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  $8.6 \times 10^8$  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/

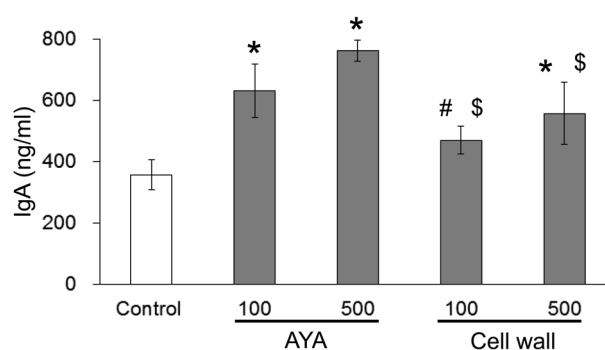


Fig. 2. IgA production level of the PP cells induced by a suspension of powdered, killed *L. plantarum* AYA or a cell wall fraction. The PP cells ( $10^6$  per well) from BALB/c mice were cultured with a suspension of powdered, killed *L. plantarum* AYA (100 or 500 µg/ml) or a cell wall fraction (100 or 500 µg/ml). Supernatants were collected after 72 hr, and IgA concentrations were measured using ELISA. The data represent the mean  $\pm$  SD ( $n = 4$ ). \* $p < 0.05$  versus the control; # $p < 0.05$  versus 100 µg/ml *L. plantarum* AYA; \$ $p < 0.05$  versus 500 µg/ml *L. plantarum* AYA (Tukey test).

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.



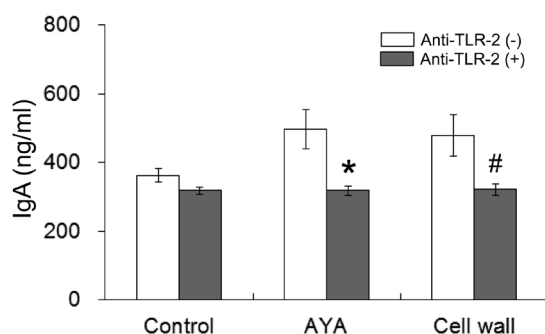


Fig. 3. The effect of the anti-TLR-2 antibody on the IgA production level of PP cells. PP cells ( $10^6$  per well) from BALB/c mice were cultured with *L. plantarum* AYA (500  $\mu$ g/ml) and its cell wall fraction (500  $\mu$ g/ml) with (+) or without (-) a monoclonal anti-TLR-2 antibody (5  $\mu$ g/ml). Supernatants were collected after 72 hr and IgA concentrations were measured using ELISA. The data represent the mean  $\pm$  SD ( $n = 4$ ). \* $p < 0.05$  versus *L. plantarum* AYA without anti-TLR-2; # $p < 0.05$  versus the cell wall fraction without the anti-TLR-2 antibody (Tukey test).

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  $\mu$ g/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). Therefore, it was confirmed that the cell wall fraction of *L. plantarum* AYA enhances the IgA level via TLR-2. It is likely that the peptidoglycans [14–16] and lipoteichoic acid [15–17] in the cell wall mediate this activity. Further research is required to confirm this assumption.

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.

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