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Effect of Lactic Acid Bacteria on Intestinal *E. coli* in *Caenorhabditis elegans*

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Abstract Lactic acid bacteria (LAB) prevent host organism from digestion-related illnesses, such as constipation, diarrhea, irritable bowel syndrome, and enteric infection. In this study, we investigated whether dietary LAB culture product reduced the level of viable intestinal *E. coli*, using nematode *Caenorhabditis elegans* as host animal. Among four LAB strains, *Lactobacillus plantarum* product most significantly reduced the number of *E. coli* living in the intestine of both 2-day and 7-day old adult *C. elegans*. The lower number of *E. coli* in the digestion system by LAB products consequently resulted in decrease of living *E. coli* in the excrement. LAB products led to an improvement of body growth, survival ratio and reproduction in *C. elegans*. Based on *in vitro* and *in vivo* antioxidant activity analysis, it was also suggested that LAB products reduce oxidative stress in the body.

Keywords: lactic acid bacteria, host intestinal bacteria, *Caenorhabditis elegans*, digestion, fermented foods

Introduction

Lactic acid bacteria (LAB) have been commercially manufactured and consumed in the form of fermented foods or dietary supplements for the purpose of promoting digestive health and immunity. Most LAB species show characteristic features of probiotics, including survival in the gastrointestinal (GI) tract, despite the strong acidic condition of stomach. LAB microflora inhibits the growth of other harmful and pathogenic bacteria in the GI tract of human, resulting in a stronger immunity of the digestion system (1). A wide range of human diseases is associated with a weak immune system caused by an imbalance between LAB and other bacteria in GI tract diseases (2). A relatively lower or insufficient number of *Lactobacillus* in GI tract is known to result in irritable bowel syndrome, including constipation and diarrhea. *Bifidobacterium* has also been shown to exert health promotion effects by producing vitamin B and protecting microflora against antibiotic therapy (3).

LAB produce bacteriocins to suppress the growth of pathogenic bacteria (4,5). Nisin, lactacin, sakasin, heveticin, and plantacin are representative bacteriocins which are synthesized by *Lactobacillus lactis*, *Lactobacillus acidophilus*, *Lactobacillus sake*, *Lactobacillus helveticus*, and *Lactobacillus plantarum*, respectively (6). In addition to bacteriocin, the metabolites specific to LAB include lactic acid and vitamins (7). Lactic acid is known to enhance the secretion of bile acid and to promote bowel movement, which elevates the absorption of nutrition in the digestion system (8). The acidification of intestine by lactic acid contributed to the reduction of infectious diseases caused by bacterial pathogens, including *Salmonella enterica* and *Pseudomonas aeruginosa* (9,10). In mammal and human GI tract, LAB provide vitamins that are not synthesized in the host animals, such as vitamin B_1, B_2, B_6, B_{12}, and K (11).

Caenorhabditis elegans is a small soil nematode organized with musculature, gonad, nervous system, excretory/secretory system, coelomocytes and intestine. *C. elegans* has both physical and chemical digestive systems which are comparable to that of human for the purpose of this study (12). The pharynx of *C. elegans* grinds and concentrates food and transfers it to the intestine (13,14), where digestive enzymes are released to lumen for the absorption of nutrients. *C. elegans* consume bacterial cells, and *E. coli* OP50 is generally used as a food for the worm when cultured in the laboratory.

In this study, a potential biological function of LAB product was investigated in *C. elegans,* focusing on intestinal bacterial cell activity and host animal's body growth, lifespan, and reproduction.

Materials and Methods

Strains Four strains of LAB (*Lactobacillus rhamnosus* KACC 11953, *Lactobacillus plantarum* KACC 10552 from pickled cabbage,

Lactobacillus helveticus KACC 12418 from a cheese, and *Bifidobacterium longum* KACC 20597 from infant intestine) were kindly provided by the Korean Agricultural Culture Collection (KACC, Suwon, Korea). *E. coli* OP50 strain used for *C. elegans* food and soil nematode *C. elegans* wild type strain, N2 were provided by Caenorhabditis Genetics Center (CGC, Twin Cities, MN, USA).

Preparation of LAB products MRS broth (BD, Franklin Lakes, NJ, USA) was used for the culture of LAB, *Lactobacillus* and *Bifidobacterium*. LAB products were obtained by boiling the supernatants harvested from a centrifugation of culture broth of each LAB cells with an optical density of 1.0 at 600 nm. For the control of LAB products, *E. coli* OP50 culture product was prepared by the same procedure. The pH of LAB and *E. coli* product was adjusted at pH 7.0 using 0.1 N HCl and NaOH solution. Before mixing with *E. coli* OP50, LAB and *E. coli* products were filtered using a membrane with 0.2 μm-diameter pores.

***C. elegans* culture** Nematode *C. elegans* strains were cultured as previously described (15). In order to synchronize the developmental stage of worms, eggs from gravid adults were prepared using a solution containing 1.5 M NaOH and 1.5% NaOCl. L1 larvae were obtained on NGM (nematode growth media) agar plates after eggs were washed with M9 buffer and were allowed to hatch overnight. To feed worms LAB products, *E. coli* OP50 cell pellets prepared from centrifugation of 150 μL of culture (O.D.=0.5 at 600 nm) were suspended with 50 μL of LAB product, which was dropped onto a NGM agar plate in a 30 mm petri dish.

Measurement of undigested *E. coli* in intestine and excrement Adult *C. elegans* fed with each LAB product for indicated days at 20°C were washed 3 times using M9 buffer (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, and 1 mL 1 M $MgSO_4$ in 1 L of H_2O), and were then disrupted in a petri dish containing 500 μL M9 buffer by cutting worm bodies using a platinum wire. The M9 buffer containing OP50 cells from disrupted worm bodies were serially diluted with fresh M9 solution and spread on LB agar plates. *E. coli* cells were then allowed to form colonies, which were scored as the number of viable *E. coli* OP50 in *C. elegans* intestine. In order to score the number of living bacterial cells in excrements, the adult worms fed LAB products for 2 or 7 days at 20°C were transferred onto NGM agar plates without bacterial lawn, and were allowed to undergo a hunger stage for 12 h. After 1 h of feeding OP50, *C. elegans* worms were washed 3 times with M9 buffer and were placed on LB agar plates. The colonies of OP50 cells excreted by *C. elegans* for 1 h were scored for the number of living *E. coli* OP50 in the excrement.

Analysis of body size, life span, and reproduction Photographs of 3 day-old adult *C. elegans* fed OP50 supplemented with LAB products were taken under a stereomicroscope (Olympus, Tokyo, Japan) with a digital camera attached, which were analyzed by computer program, Image J (NIH, Bethesda, MD, USA) to measure the length of worms. For the investigation of lifespan, 100 L4 larvae of *C. elegans* were placed on 10 NGM agar plates containing a bacterial lawn supplemented with LAB product. Living worms were scored and transferred to new plates with a fresh bacterial food supply every day (15). The percentage of survival was obtained by calculating the ratio of living to total animals. The effect of LAB product on *C. elegans* reproduction was examined by scoring total progeny eggs from each 100 parent worms for the first 5 days of adulthood.

Assessment of *in vitro* and *in vivo* antioxidant activity of LAB products DPPH free radical (Sigma-Aldrich, St. Louis, MO, USA) scavenging assay was used to examine *in vitro* antioxidant activity of extracellular LAB products. DPPH radicals are reduced by antioxidants, which change the color of DPPH from deep violet to light yellow (16). A decrease of absorbance at 517 nm indicates the scavenging of DPPH radicals, which corresponds to the strength of antioxidant activity by LAB products. Each LAB product sample was mixed and reacted with the same volume of 0.3 mM DPPH dissolved in ethanol for 1 h at room temperature. The absorbance of the reaction mixture was measured at 517 nm using a UV/VIS spectrophotometer Evolution 300 (Thermo Scientific, Madison, WI, USA). The inhibition percentage of each LAB product was determined by comparison of the absorbance of reaction mixture containing DPPH and MRS medium (blank). Radical scavenging activity was evaluated from the following equation:

$$\% \text{ Inhibition}=\{(A_0-A)/A_0)\}\times 100$$

where, A_0 and A are the absorbance of the sample and blank, respectively.

Reactive oxygen species (ROS) generated in the body of *C. elegans* were monitored using a fluorescence dye DCF, which was formed by ROS-induced oxidation of 2',7'-dichlorodihydrofluorescein ($DCFH_2$). Adult worms fed OP50 or LAB product for 7 days at 20°C were washed 3 times with M9 and were consequently incubated in 0.2 μM $DCFH_2$-diacetate in Hank's solution for 30 min at 20°C. The worms were transferred onto 5% agar pad on a slide glass and were paralyzed by dropping 5 μL of 5 mM levamisole on the worms. A fluorescence microscope Axio Imager 2 (Carl-Zeiss, Jena, Germany) with excitation (485 nm) and emission (530 nm) filters was used to observe the green fluorescence of 2',7'-dichlorofluorescein (DCF) activated by ROS in *C. elegans*.

Assessment of polyphenol content in LAB products Polyphenols in LAB and *E. coli* products were determined using Folin-Ciocalteu reagent (17). Each sample diluted with deionized water was mixed with Folin-Ciocalteu reagent and 10% Na_2CO_3 (1:1:2). The mixture was centrifuged at 15,000×*g* for 15 min, and the supernatant was filtered through filter paper (Whatman No. 1; Sigma-Aldrich). The absorbance of mixture solution was measured at the wavelength of 765 nm using UV/Vis spectrophotometer (Evolution 300; Thermo Scientific). Each sample was analyzed twice with duplicates. Results

were expressed as the percentage normalized by absorbance of *E. coli* culture product, ECP.

Results and Discussion

Reduction of undigested living *E. coli* in the Intestine Nematode *C. elegans* were cultivated on their foods, *E. coli* OP50. Oral-administered *E. coli* cells were ground in the pharynx of *C. elegans* and enzymatically digested in intestine lumen under acidic conditions. Despite such physical and enzymatic digestion system, undigested and living food bacteria are frequently found in the intestine of *C. elegans*. The effect of LAB product on the promotion of digestion efficiency was demonstrated by measuring the number of undigested living *E. coli* in the intestine (18). To analyze the number of living *E. coli* cells, the colonies were counted at 1 day after incubation of bacterial cells from *C. elegans* intestine on LB agar plates. The number of undigested food bacteria was drastically reduced in the animals with pretreatment of LAB products for 2 days compared to that from the worms with *E. coli* products (Fig. 1A). In particular, LHP (*Lactobacillus helveticus* product) and LPP (*Lactobacillus plantarum* product) showed a significant increase of digestion efficiency in *C. elegans* resulting in a small number of living cells in their intestine. The digestion efficiency of *C. elegans* was diminished with aging. The number of undigested *E. coli* in a 2-day old adult was 1.4×10^3 CFU/mL, however; a 7-day old adult contains 1.2×10^4 CFU/mL living bacteria in the body. LAB products exerted their digestion-promoting activity in the 7-day old adults, resulting in an approximate 35% reduction of undigested *E. coli* in the gut (Fig. 1B). In both 2 and 7 days old adults, the extracellular product from *Lactobacillus plantarum* showed the strongest efficacy among those 4 different LAB.

Reduction of undigested living *E. coli* in excrement Living bacteria in the intestine lumen are released out of body through excrement of *C. elegans* (19). Given that LAB products improved the digestion of bacterial foods in *C. elegans* by reducing the number of *E. coli* in the intestine, it was expected that the undigested and living bacterial cells might be reduced in the excrement of animals. In order to examine whether LAB products reduced the loss of living bacterial cells through feces, the shedding assay to score the number of *E. coli* released from worms was carried out using 2-day old adults. The animals with experience of exposure to LAB products for 2 days from L4 stage excreted fewer living bacteria than control worms treated with *E. coli* product (Fig. 2A). This digestion-promotion effect to reduce living bacterial food in the excrements was also exerted in the older adults fed with LAB products for 7 days (Fig. 2B). Although there was variation of efficacy level among the products, LPP showed the most enhanced digestion efficiency. These results demonstrate that extracellular products of LAB cultures have a potential for improving animal digestion efficiency by improving digestion in the intestine.

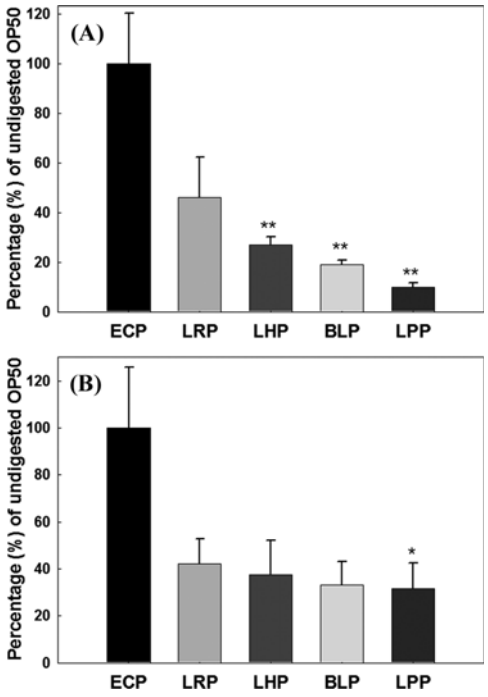

Fig. 1. Effect of LAB products on the reduction of living *E. coli* in intestine of 2-day (A) and 7-day (B) old adult *C. elegans* (N=50, **p*<0.05 and ***p*<0.01) (ECP: *E. coli* product, LRP: *Lactobacillus rhamnosus* product, LHP: *Lactobacillus helveticus* product, BLP: *Bifidobacterium longum* product, LPP: *Lactobacillus plantarum* product)

Improvement of body growth, survival and reproduction Colonization or survival of undigested *E. coli* in the body interferes with digestion of food bacteria or uptake of nutrition in the animal. The enhancement of digestion efficiency implies an increase of nutrition transport from a gut to other organs in the body, which contributes to animal growth and reproduction. Body length of *C. elegans* was measured by image analysis using pictures of 2-day old adults fed with LAB or *E. coli* products for 3 days from L4 stage. As shown in Fig. 3A, the number of animals with larger body size (1.4 mm) increased when the worms were grown on food mixed with LAB products. The body size of worms fed with LPP was 1.32±0.008 mm on average, while that of control animals with ECP (*E. coli* OP50 product) was 1.23±0.01 mm.

In a previous study, the lifespan of *C. elegans* was prolonged when the worms were fed with LAB (20). In order to examine whether the extracellular product of LAB displayed a similar effect on lifespan, we measured the survival ratio of *C. elegans* every day in their adulthood until death (Fig. 3B). Unlike LAB cells, the extracellular culture products did not extend the lifespan of *C. elegans*. However, LAB products improved the survival ratio of adults with age from 8 to 12 days in comparison with animals without LAB product. The average survival ratio of 11-day old animals fed with LAB products was 56.75%, whereas the mean viability of control group fed with ECP was 41% at the same age.

We have also examined whether the extracellular LAB products

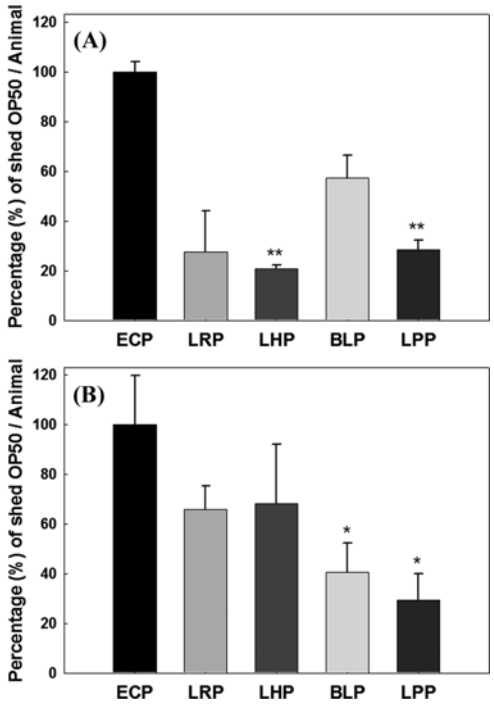

Fig. 2. Effect of LAB products on the reduction of living *E. coli* in excrement from 2-day (A) and 7-day (B) old adult *C. elegans* (N=25, **p*<0.05 and ***p*<0.01) (ECP: *E. coli* product, LRP: *Lactobacillus rhamnosus* product, LHP: *Lactobacillus helveticus* product, BLP: *Bifidobacterium longum* product, LPP: *Lactobacillus plantarum* product)

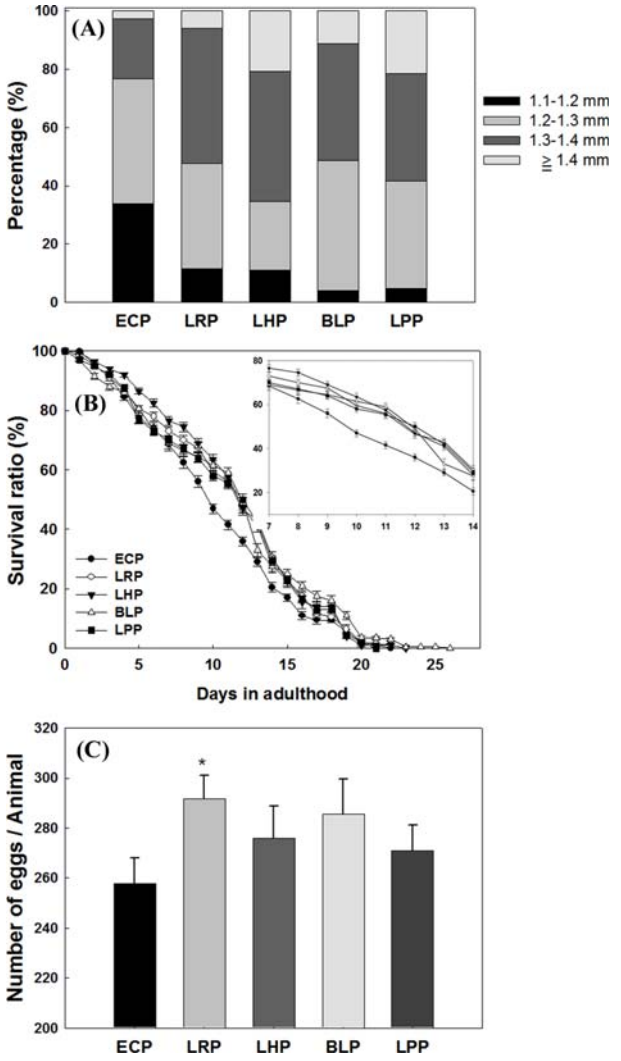

Fig. 3. Effects of LAB products on the increase of body growth (A, N=430), survival ratio (B, N=1,000), and progeny reproduction (C, N=1,200) in adult *C. elegans* (**p*<0.05) (ECP: *E. coli* product, LRP: *Lactobacillus rhamnosus* product, LHP: *Lactobacillus helveticus* product, BLP: *Bifidobacterium longum* product, LPP: *Lactobacillus plantarum* product)

were able to enhance the reproduction capability of adult *C. elegans*. In addition to body growth, the reproduction of progenies has been known to require large energy consumption in animals. By scoring all the eggs laid by adults, it was found that LAB products increased the reproduction capability resulting in a larger brood size (Fig. 3C). Among those products, LRP exerted the strongest reproduction-promotion activity.

Antioxidant activity of LAB products It was reported that probiotic bacteria reduced oxidative stress of host animals by producing antioxidants in GI tracts (21). We have examined whether LAB extracellular products contain such antioxidants by measuring DPPH free radical scavenging activity. LAB products exerted a strong antioxidant effect on DPPH free radicals resulting in a 70% (average of 4 different LAB products) inhibition rate, whereas *E. coli* product showed just a 10% radical scavenging activity (Fig. 4A).

Given that LAB products displayed *in vitro* antioxidant activity, we have also investigated their *in vivo* activity to reduce oxidative stress in the body of *C. elegans* using ROS (reactive oxygen species)-induced fluorescence dye, dichlorofluorescein diacetate (DCF-DA). As shown in Fig. 4B, the bright green fluorescence from DCF was detected in whole body of *C. elegans* when the animals were fed with *E. coli* product. However, the fluorescence, indicating ROS level, was reduced in the worms fed with LAB products (Fig. 4C-4F). The ROS level was quantitatively measured using a computer program for image analysis. Among the LAB products, LHP (*Lactobacillus helveticus* product) significantly diminished oxidative stress in the animal to 1/30 that of ECP (Fig. 4G). These results demonstrate that the antioxidants in LAB extracellular products retained their activity in the body despite the animal's digestion process.

A few studies about the beneficial health effects of LAB have been described using nematode *C. elegans* system. Co-feeding or pre-feeding of LAB increased the survival ratio or lifespan of *C. elegans* when the worms were exposed to pathogenic bacteria or oxidative stress (18). Although these studies insufficiently confirmed whether LAB cells were alive in the GI tract of *C. elegans*, oral-administered LAB enabled the host animals to inhibit the growth of pathogenic bacteria and oxidative stress. Instead of bacterial cells itself, we herein investigated the effects of extracellular small molecule products from four LAB cultures on digestion, excrement, lifespan,

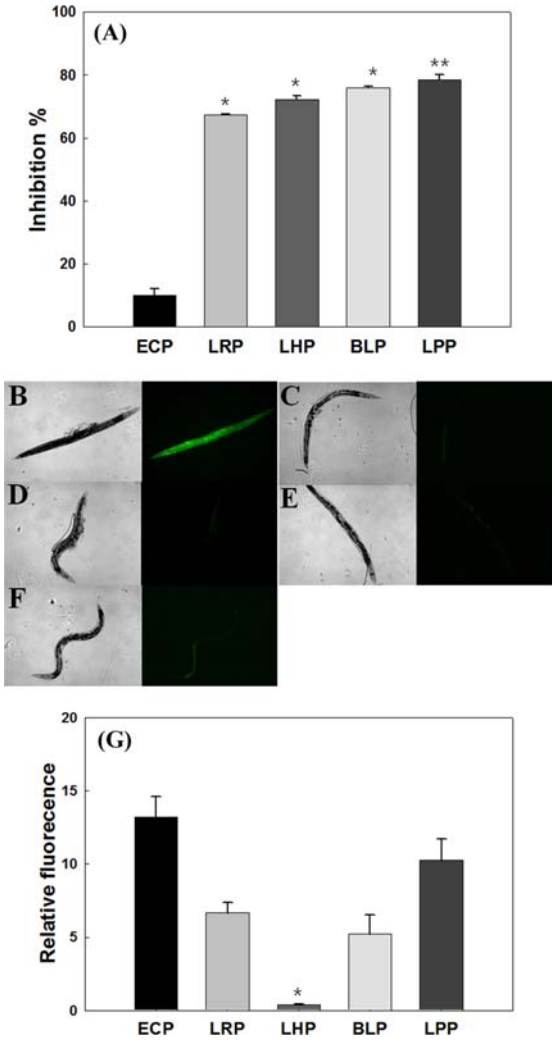

Fig. 4. Scavenging of DPPH free radicals *in vitro* (A) and reduction of ROS level (B-G) in *C. elegans* by LAB products (N=15, **p*<0.05 and ***p*<0.01) (ECP: *E. coli* product, LRP: *Lactobacillus rhamnosus* product, LHP: *Lactobacillus helveticus* product, BLP: *Bifidobacterium longum* product, LPP: *Lactobacillus plantarum* product)

reproduction, growth, and oxidative stress of *C. elegans*. Since a large amount of protein in the extracellular products was inactivated and removed through precipitation by centrifugation after autoclave, the observed beneficial effects could mainly result from small molecules other than proteins. Although a further study has to be required to identify active components in these extracellular products, the present study describes a novel discovery with a view to the first application of extracellular LAB products to *C. elegans* systems. Given that many studies using LAB have not suggested a sufficient chemical or biological mechanism behind the animal's physiological effects, our study using extracellular products is considered meaningful to provide possible evidence that extracellular components in cell cultures make a contribution to such mechanisms of beneficial health effects in *C. elegans*.

Since we confirmed that a constant number of *E. coli* (19.75±1.04

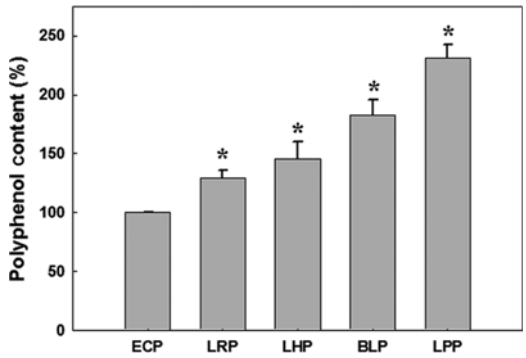

Fig. 5. Polyphenol contents in LAB products (**p*<0.01) (ECP: *E. coli* product, LRP: *Lactobacillus rhamnosus* product, LHP: *Lactobacillus helveticus* product, BLP: *Bifidobacterium longum* product, LPP: *Lactobacillus plantarum* product)

CFU/animal) was maintained in *C. elegans* intestine after the hunger stage, the measurement to score colonies of *E. coli* prepared from the gut of *C. elegans* were employed to examine the efficiency of digestion in the animals. The lower number of colonies than the control group indicates that the greater number of *E. coli* was killed and digested in the gut of animals. In previous studies, the consumption of LAB cells significantly suppressed the toxic effects of pathogenic bacteria in *C. elegans* (22). Such antibacterial activity of LAB cell consumption correlated to our result of the efficient digestion of *E. coli*, which demonstrates the contribution of extracellular products of LAB cells to the death of bacteria in the GI tract of *C. elegans*. The enhanced digestion of bacterial food in the gut consequently resulted in reduced number of living *E. coli* in the animals' excrement. Since many foodborne illnesses are distributed through the contact of excrement containing living pathogenic bacteria, the reduction of living cells in defecation using LAB products has a potential to reduce the transmittance of such infectious diseases.

Among four LAB products, LPP (*Lactobacillus plantarum* product) showed the most significant enhancement of digestion activity, which drastically reduced the living bacterial cell concentration in both gut and excrement. The improvement in digestion by LPP products consequently led to an increase of body size growth in adult *C. elegans*. In a previous study, the body sizes of *C. elegans* fed LAB significantly decreased during the first 3 days after the food bacteria were switched from *E. coli* OP50 to *bifidobacteria* (20). Although the mechanism of bifidobacteria on body size was not proposed in detail, a different nutritional factor between *E. coli* and bifidobacteria might result in the smaller body growth of *C. elegans*. However, in this study, the worms were not fed LAB cells instead of *E. coli*. Since only the extracellular products of LAB cells were supplemented to the food, *E. coli*, both groups of worms consumed the same bacterial strain, OP50. No adaptation process of animals to new food was required in our system, which resulted in the determination of an improvement of body growth by the help of LAB products.

Among the four LAB products, LPP also exerted the strongest *in*

vitro antioxidant activity in DPPH free radical scavenging assay. It has recently reported that *Lactobacillus plantarum* strains isolated from Chinese fermented foods showed a 53.05% inhibition rate in DPPH scavenging activities (23), which was about 20% lower than that of LPP in our present study. Some previous studies described that the supplementation of antioxidants increased viability, reproduction and growth of *C. elegans* due to an enhancement of immunity (24). Since LPP showed the most significant effects on digestion and growth, the health beneficial effects by LAB products was strongly considered to be dependent on the reduction of oxidative stress in *C. elegans*.

The most widely used food strain of *C. elegans*, *E. coli* OP50, has been shown to induce a pathogenic effect in old adult worms (20). In the present study, the number of living *E. coli* OP50 in the intestine increased from 1.4×10^3 CFU/mL (2-day old adult) to 1.2×10^4 CFU/mL (7-day old adult). This result implied that OP50 was not digested as food but was colonized in *C. elegans* like other pathogenic bacteria. In a previous study, it was also reported that the worms fed dead/non-viable OP50 survived longer than those fed live bacteria. Given that the digestion in *C. elegans* indicates the killing of bacteria, the improved digestion by LAB product corresponds to the increased disruption of bacterial cells in the digestion system. Such antibacterial activity of LAB products reduced the pathogenic effect of food bacteria, which led to an increase in the survival ratio of adult *C. elegans*. Although a further study about an exact mechanism behind the disruption of *E. coli* OP50 by LAB should be completed, both direct and indirect killing mechanism are possible as shown in some of previous studies (18,20,22). LAB showed to produce anti-bacterial components that can directly kill bacterial cells and also increase host immune activity especially on pathogenic bacteria by inhibiting the colonization of cells.

In order to illuminate what constituents of LAB took part in such beneficial effects on digestion system in *C. elegans*, total polyphenol content of LAB was assessed using Folin-Ciocalteu reagent. As shown in Fig. 5, the polyphenol contents of LAB were significantly larger than that of *E. coli* OP50 product. Results demonstrate that some of LAB polyphenols modulates the host immune system of *C. elegans* resulting in reduction of living bacterial cells in the gut and feces. Recently, it was also reported that polyphenol compounds including sinensetin and eupatorin of plant extract strengthened *C. elegans* immune system against pathogenic *S. aureus* (25).

Although further studies about which constituents of LAB products display antibacterial and antioxidant effects in the animal body, the proposed results strongly suggest that oral-administrated extracellular molecules originating from LAB cultures promote the health of host digestion systems, without the uptake of living LAB cells.

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Disclosure The authors declare no conflict of interest.

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