Antioxidant status and gut microbiota change in an aging mouse model as influenced by exopolysaccharide produced by *Lactobacillus plantarum* YW11 isolated from Tibetan kefir

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ABSTRACT

This study investigated the effect of exopolysaccharide (EPS) produced by *Lactobacillus plantarum* YW11 on the oxidative status and gut microbiota in an aging mouse model induced with D-galactose. The in vitro assay of the antioxidant activity of the EPS showed concentration-dependent (0.25–3.0 mg/mL) activities. At 3.0 mg/mL, the EPS reached the highest scavenging activities with half maximal inhibitory concentration values against hydroxyl radicals at 75.10% and 1.22 mg/mL, superoxide anion at 62.71% and 1.54 mg/mL, 2, 2-diphenyl-1-picrylhydrazyl at 35.11% and 0.63 mg/mL, and the maximal chelating rate on ferrous ion and the half-maximal chelating concentration of the EPS at 41.09% and 1.07 mg/mL, respectively. High doses of EPS (50 mg/kg per day) effectively relieved the oxidative stress in the aging mice with increased levels of glutathione peroxidase, superoxide dismutase, catalase, and total antioxidant capacity in mice serum by 21.55, 33.14, 61.09, and 38.18%, respectively, and decreased malondialdehyde level from 11.69 to 5.89 mmol/mL compared with those in the untreated aging mice model. The analysis of pyrosequencing sequence data from the gut microbiota revealed that the EPS could recover the microbiota diversity and phylotypes decreased or eliminated by the D-galactose treatment. The EPS could selectively decrease the abundance of *Flexispira* (37.5 fold), and increase the abundance of *Blautia* (36.5 fold) and *Butyricicoccus* (9.5 fold), which correspondingly decreased the content of nitrogen oxides to 9.87% and increased the content of short-chain fatty acids by 2.23 fold, thereby improving the oxidative and health conditions of the host intestinal tract. Further correlation analysis of core-microbiota variation induced by different treatments showed a strong correlation with oxidative phenotypes [catalase, goodness of prediction

(Q^2) = 0.49; total antioxidant capacity, Q^2 = 0.45; nitrogen oxides, Q^2 = 0.67; short-chain fatty acids, Q^2 = 0.55]. The fermented milk with *L. plantarum* YW11 containing EPS also showed favorable antioxidant and gut microbiota regulating activities. The present finding provided new insights into the functional mechanism of probiotics bioactivity.

Key words: antioxidant, exopolysaccharide, fermented milk, gut microbiota

INTRODUCTION

Oxidative stress in living organisms can be caused by an increased load or accumulation of reactive oxygen species (ROS), which is generally associated with various physiological malfunctions and diseases, including immunomodulation (Park et al., 2015), aging (Li et al., 2016b), apoptosis (Formichi et al., 2016), cancer (Glorieux et al., 2016), and Parkinson's and Alzheimer's diseases (Bhattacharjee et al., 2016; Hamulakova et al., 2016). The oxidative stress can be relieved by the counteraction of defense system with ROS through neutralization with antioxidants (Wen et al., 2015). Functional dietary antioxidant ingredients originating from fermented foods play an important role in preventing occurrence of diseases associated with oxidative stress by directly inhibiting of lipid peroxidation, scavenging ROS, and regulating intestinal environment (Coskun Cevher et al., 2015). These dietary antioxidants mainly include bioactive peptides (Ohata et al., 2016), microbial enzymes, and metabolites (Mishra et al., 2015).

Kefir has been well known for its many health-benefiting activities, including antioxidative, antimicrobial, and anticarcinogenic activity (Ahmed et al., 2013). Exopolysaccharide (EPS) isolated from this traditional product were shown to possess antioxidant (Luang-In and Deeseenthum, 2016), anti-inflammatory, and angiogenic activities (Prado et al., 2016), as well as the ability to modulate metabolism of key microorganisms in gut microbiota (Serafini et al., 2014). The EPS from *Weissella cibaria* and *Pediococcus pentosaceus* were found to

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be highly resistant to gastric and intestinal digestions and capable of selective enhancement of beneficial gut bacteria, in particular *Bifidobacteria* (Hongpattarakere et al., 2012). The EPS produced by *Lactobacillus plantarum* DM5 showed a low digestibility by artificial gastric juice and inhibitory effect on the growth of nonprobiotic bacteria, such as *Enterobacteriaceae* (Das et al., 2014). These results indicated that EPS were similar to prebiotics and effective in regulation of gut microbiota in vitro (Serafini et al., 2014). As many oxidant-related diseases have been proven to relate to structure and metabolite change of microbiota, including colon cancer (Sears and Garrett, 2014), cardiovascular disease (Siti et al., 2015), diabetes (Karunakaran and Park, 2013), obesity (Nistala et al., 2014), and aging (Claesson et al., 2012), these findings suggest a possible mechanism of microbial EPS improving oxidative status of the host by regulating microbiota to selectively inhibit or eliminate microorganisms related to oxidative stress. Until now, the microbiota-regulatory effect of EPS produced by lactic acid bacteria (LAB) in vivo and their consequent influence on the oxidative status of host have not been studied. Considering the food-grade origin of EPS produced by LAB, determination of their microbiota-regulatory activity and health-beneficial functions on the host are important for their potential applications as functional ingredients and for further understanding the functional mechanism of LAB.

Previously, *L. plantarum* YW11 isolated from Tibetan kefir was shown to produce an EPS, which accounted for more than half amount of the polysaccharide secreted during kefir fermentation (Wang, 2015). This EPS had a molecular mass of 1.1×10^5 Da, consisting of glucose and galactose (molar ratio of 2.71:1) (Wang et al., 2015a). The present study was performed to examine the in vivo antioxidant effect of this EPS and determine its regulatory effect on the gut microbiota in correlation with the antioxidant status in an aging mouse model, in which oxidative stress was induced with D-galactose. Recovering the diversity and abundance of gut microbiota in the aging mice after administration of different doses of the EPS and the fermented milk with *L. plantarum* YW11 was investigated using microbial diversity analysis. A possible correlation of the gut microbiota change with the oxidative status of mice was studied using regression analysis.

MATERIALS AND METHODS

Strains and Culture

Lactobacillus plantarum YW11 was isolated from Tibetan kefir grains and kept in the dairy food processing laboratory of Beijing Technology and Business

University. *Lactobacillus plantarum* YW11 was cultured at 37°C for 18 h in skim milk medium prepared by reconstituting 10% skim milk powder (Fonterra, Auckland, New Zealand) in deionized water under nonshaking conditions. Fermented milk used in the in vivo experiment was prepared by fermenting the pasteurized (85°C, 5 min) 10% (wt/wt) skim milk with only *L. plantarum* YW11 at 37°C for 18 h, with the inoculation rate of 1% and viable count of *L. plantarum* YW11 at 8.41 log cfu/mL.

Preparation of EPS

Lactobacillus plantarum YW11 was cultured in 10 L of pasteurized (85°C, 5 min) skim milk medium at 37°C for 18 h. The EPS produced by the strain was then isolated and prepared using the method previously reported (Wang et al., 2015a). Briefly, after fermentation, 80% (wt/vol) trichloroacetic acid (TCA) was added to achieve the 4% (wt/vol) trichloroacetic acid concentration. The mixture was stirred at room temperature for 2 h, and then centrifuged at $12,000 \times g$ for 30 min at 4°C. The cold ethanol (4°C) was added to the supernatant to achieve 70% concentration and then kept at 4°C overnight. The pellet of crude EPS was collected after centrifugation at $12,000 \times g$ for 30 min at 4°C. For purification, the EPS was dissolved in distilled H₂O (20 mg/mL) and fractionated with a diethylaminoethyl cellulose column. Peak fractions containing EPS were lyophilized and fractionated by Sepharose CL-6B column (GE Healthcare, Pittsburgh, PA). The fraction of pure EPS was lyophilized into powder and stored at -20°C for further use.

In Vitro Antioxidant Activity

Preparation of EPS Solution. The EPS solution was prepared by completely dissolving EPS in PBS (pH 7.2) to achieve concentrations of 0.25, 0.5, 1.0, 2.0, and 3.0 mg/mL for further tests. An ascorbic acid solution of the same concentrations was used as a positive control.

Determination of Hydroxyl Radical Scavenging Activity. The hydroxyl radical scavenging activity was examined using the method of Wang et al. (2009) with some modifications. Briefly, the reaction mixture containing 2.0 mL of FeSO₄ (0.5 mM), 1.0 mL of brilliant green (0.435 mM), 1.5 mL of H₂O₂ (3.0%, wt/vol), and 1.0 mL of EPS solution of different concentrations (0.25, 0.5, 1.0, 2.0, and 3.0 mg/mL) was incubated at 25°C for 20 min, and the absorbance was measured at 624 nm. The scavenging activity was calculated as scavenging activity (%) = $[(A_{\text{sample}} - A_{\text{blank}})/(A_{\text{control}} - A_{\text{blank}})] \times 100$, where A_{sample} is the absorbance of the

mixture containing EPS and Fenton solution, which was prepared by mixing previously mentioned solutions of FeSO_4 and H_2O_2 , A_{blank} is the absorbance of mixture without EPS, and A_{control} is the absorbance of mixture without EPS and Fenton solution.

Determination of 2,2-diphenyl-1-picrylhydrazyl Radical Scavenging Activity. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity of EPS was determined using the method of Ye et al. (2012). Briefly, 1.0 mL of EPS solution of different concentrations (0.25, 0.5, 1.0, 2.0, and 3.0 mg/mL) was added to 2.0 mL of ethanolic DPPH radical solution (0.05 mM). After thorough mixing, the mixture was incubated at 25°C for 30 min in a lucifugal cabinet. The mixture was centrifuged at $8,000 \times g$ for 10 min at 4°C, and the absorbance of the supernatant was measured in triplicate at 517 nm. The scavenging activity (%) was calculated as $[1 - (A_{\text{sample}} - A_{\text{blank}})/A_{\text{control}}] \times 100$, where A_{sample} is absorbance of the mixture containing EPS and DPPH solution; A_{control} is absorbance of the mixture containing only deionized water and DPPH solution; and A_{blank} is absorbance of mixture containing only ethanol and EPS.

Determination of Superoxide Anion Radical Scavenging Activity. The scavenging activity of superoxide radical was determined using the method of Giese et al. (2015). Briefly, 1.0 mL of EPS solution of different concentrations (0.25, 0.5, 1.0, 2.0, and 3.0 mg/mL) or deionized water as a control was mixed with 3.0 mL of 0.05 M Tris-HCl buffer (pH 8.2) and incubated at 25°C for 10 min. Then, 40 μL of 45 mM pyrogallol was added to the mixture and incubated for 5 min. The absorbance of the resulting solution was measured in triplicate at 325 nm. The scavenging ability (%) was calculated as $[1 - (A_{\text{sample}}/A_{\text{control}})] \times 100$, where A_{sample} is the absorbance of the sample containing EPS and A_{control} is the absorbance of the sample without EPS.

Ferrous Ion Chelating Ability. Ferrous ion chelating activity was investigated using the method of Chen et al. (2015a). Briefly, the reaction mixture containing 0.2 mL of different concentrations of samples (0.25, 0.5, 1.0, 2.0, and 3.0 mg/mL), FeCl_2 (0.1 mL, 2

mM), and ferrozine (0.4 mL, 5 mM) was shaken well and incubated for 10 min at room temperature. The absorbance of the mixture was measured at 562 nm against a blank (the same volume of PBS). Ethylene diamine tetraacetic acid was used as the standard positive control.

Animal Experiments

Animal Grouping and Treatment. The animal experiments in our study were performed at the animal facilities of a local hospital in Beijing, China, and conducted strictly according to the Provisions and General Recommendations of Chinese Experimental Animal Administration Legislation. After 1 wk of acclimatization, 48 male Institute of Cancer Research mice (8 wk old, 23–25 g of BW) were randomly divided into 6 groups (8 mice per group); 2 mice in each group were used as a backup. Daily treatments were performed according to Table 1. The mice in the treatment groups were subcutaneously injected with 10 mL of 5% (wt/wt) D-galactose solution per kg of BW per day to induce oxidative stress; the mice in the normal group were injected with 10 mL of 0.9% (wt/wt) normal saline solution per kg of BW per day by oral gavage. The mice in the aging group received 20 mL of 0.9% (wt/wt) normal saline solution per kg of BW per day by oral gavage and served as a negative control. The mice in the vitamin C (VC) group received 20 mL of 1 mg/mL ascorbic acid solution per kg of BW per day and served as a positive control. The mice in the low-dose (LD) group received 20 mL of 1 mg/mL EPS solution per kg of BW per day; the mice in the high-dose (HD) group received 20 mL of 2.5 mg/mL EPS solution per kg of BW per day; and the mice in the fermented milk (FM) group received 20 mL of fermented milk per kg of BW per day. The fermented milk contains 8.41 log cfu/mL of viable *L. plantarum* YW11 strains. The whole process lasted for 12 wk.

Detection of Antioxidant Activity in Serum. After the mice were anesthetized using diethyl ether (Sinopharm Chemical Reagent Co. Ltd., Beijing, Chi-

Table 1. Groups of experimental mice and their daily treatments¹

Group	Oral gavage (/kg of BW/day)	Subcutaneous injection (/kg of BW/day)
Normal	20 mL of NSL	10 mL of NSL
Aging	20 mL of NSL	10 mL of 5% D-galactose solution
Ascorbic acid (VC)	20 mL of 1 mg/mL ascorbic acid solution	10 mL of 5% D-galactose solution
Low dose (LD)	20 mL of 1 mg/mL EPS solution	10 mL of 5% D-galactose solution
High dose (HD)	20 mL of 2.5 mg/mL EPS solution	10 mL of 5% D-galactose solution
Fermented milk (FM)	20 mL of fermented milk	10 mL of 5% D-galactose solution

¹VC = group of mice treated with vitamin C; LD = group of mice treated with a low dose of exopolysaccharide (EPS); HD = group of mice treated with high dose of EPS; FM = group of mice treated with fermented milk; NSL = 0.9% normal saline.

na), blood (about 2 mL) was collected from the orbital vein plexus. The serum was prepared by centrifuging at $3,000 \times g$ for 15 min at 4°C. Serum was stored at -70°C for further analysis. Enzyme-linked immunosorbent assay kits (Nanjing Jiancheng co., Nanjing, China) were used to determine the malondialdehyde (MDA) level, and the activities of glutathione peroxidase (GSH-Px) activity, total antioxidant capacity (T-AOC), catalase (CAT), and superoxide dismutase (SOD) in the serum following the manufacturer's protocol (<http://www.njjcbio.com/product.asp?cid=27>). Briefly, the MDA level was measured by the 2-thiobarbituric acid method and expressed as nanomoles per milliliter in serum. The enzymatic activities of GSH-Px, T-AOC, CAT, and SOD were determined using the methods of GSH-H₂O₂ reaction system, ferric-reducing/antioxidant power reaction system, CAT-H₂O₂ reaction system, and xanthine oxidase-xanthine reaction system, respectively. The enzymatic activities were expressed as units per milliliter in serum.

Fecal DNA Extraction and Pyrosequencing. Genomic DNA was extracted from fecal samples by bead beating and using a 51504-QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). In brief, approximately 0.22 g of frozen feces were added to a 2-mL Eppendorf tube containing 1 mL of InhibitEX buffer of the kit and 0.3 g of glass beads (0.1 mm, Biospec Products Inc., Bartlesville, OK). After sufficient homogenization by vortexing for 1 min, bead beating was performed for 1 min at full speed. Bacterial DNA was extracted using proteinase K treatment and subsequent purification following the manufacturer's protocol (<https://www.qiagen.com/cn/shop/sample-technologies/dna/genomic-dna/qiaamp-dna-stool-mini-kit/#resources>). The extracted DNA from each sample was used as the template to amplify a 250 to 300 bp fragment covering the V4 region of 16S rRNA genes. Sequences of the fragment were obtained by paired-end sequencing using MiSeq PE300 (Illumina Inc., San Diego, CA). Polymerase chain reaction amplification, pyrosequencing of PCR amplicons, and quality control of raw data were performed as described previously (Wang et al., 2012).

Bioinformatics and Multivariate Statistics

Pyrosequencing sequences of high quality (excluding truncated sequences below an average quality of 20 over a 25-bp sliding window, sequences with trimmed reads having less than 75% of their original length, and sequences with reads contaminated by adapter, ambiguous bases and reads with 10 consecutive same base) were compared and clustered using cluster database at high identity with tolerance with 99% or

higher similarity. The longest sequence in each cluster was aligned against the Greengenes database (DeSantis et al., 2006) to construct a neighbor-joining tree. Operational taxonomic units (OTU) with higher than 97% similarity were delineated using the method of Schloss and Handelsman (2005), in which a distance-based OTU and richness (DOTUR) program was used and sequences were assigned to OTU according to their distance level in neighbor algorithm. The coverage of OTU was calculated as coverage = $1 - n1/N$, where $n1$ is the number of OTU containing only 1 sequence and N is the numbers of OTU in the sample. The principal coordinate analysis (PCoA) was applied to determine the differences in OTU composition of different groups and key factors responsible for the difference using a relative abundance value. The distance of samples in the PCoA plot stood for the similarity of species composition between the samples. The software used in this step was package ade4 of R software (v3.1.1; <https://www.r-project.org/>).

A redundancy analysis (RDA) was completed with CANOCO (Microcomputer Power, Ithaca, NY) according to the software manual (Braak and Smilauer, 2002). Briefly, species and environmental data were input into CANOCO software. In the method-selection inspection, the lengths of gradient (2.77) in the detrended correspondence analysis was lower than 3.0, suggesting that the method of RDA was more suitable for data analysis than for canonical correspondence analysis. Parameters of RDA were set as "Focus scaling on" = "Symmetric," "Species scores" = "Divide by standard deviation," "Centering and standardization samples" = "none," "center by species." The significance of RDA analysis was calculated using the Monte Carlo permutation procedure with 499 random permutations. The OTU with at least 10% of variability in their values explained by the first axis in RDA were used in further analysis as key OTU. The heat map of key OTU was plotted according to the method described by Perez-Llamas and Lopez-Bigas (2011). Briefly, the key OTU were arranged according to their neighbor-joining positions, and the relative abundance data of the key OTU were normalized and log-transformed before building correspondence to the color squares using the software of MeV (Dana-Farber Cancer Institute, Boston, MA).

Possible correlation of gut microbiota structure with the oxidative status of mice was predicted using the partial least square regression (PLS) model. The performance of regression model was examined using leave-one-out cross-validation (Wold et al., 2001) and Pearson correlation coefficient and *P*-value between predicted and detected values. The analysis was performed using XL stat2015 software (Addinsoft, New York, NY).

Nitrogen Oxides Determination

The concentration of nitrogen oxides (NO_x) in the feces was determined using a Griess reaction (Green et al., 1982). Specifically, 0.5 mL of the supernatant of fecal samples was prepared by the homogenization of feces (0.2 g) with 1.0 mL of PBS (pH 7.4) and centrifugation at $3,000 \times g$ for 5 min at 4°C . The supernatant was mixed with 0.25 mL of 0.3 M NaOH and incubated at 25°C for 5 min; 0.25 mL of 10% (wt/vol) ZnSO_4 was then added and centrifuged at $12,000 \times g$ for 10 min at 4°C for the elimination of protein. The nitrate content in supernatant was determined using the spectrophotometric method before reduction into nitrite using VaCl_3 . The standard curve was plotted with sodium nitrite solution of 0, 5, 10, 20, 50, and 100 $\mu\text{mol/L}$.

Short-Chain Fatty Acids Determination

Fecal samples were homogenized by mixing with 0.9 mL of 0.01 M PBS. The mixture was centrifuged at $8,000 \times g$ for 10 min at 4°C . The supernatant was mixed with one-sixth volume of 7 M H_2SO_4 and extracted with 1 mL of ethyl ether by vibration for 5 min. The concentrations of acetic, propionic, butyric, and isobutyric acid were determined using the GC method reported by (Wu et al., 2016).

Statistical Analysis

All chemical measurements were done in triplicate samples on duplicate trials and data were presented as means $\pm$ standard deviation of means. Statistically significant differences were determined by one-way ANOVA followed by Tukey's tests in SPSS 22.0 software (IBM Corp., Armonk, NY).

RESULTS AND DISCUSSION

In Vitro Antioxidant Activity

Hydroxyl, DPPH, and superoxide radicals have been widely accepted as models for evaluating ROS-related antioxidant activity (Wu and Huang, 2017). The scavenging capacities of the EPS produced by *L. plantarum* YW11 against these 3 ROS were examined using ascorbic acid as a positive control. Figure 1A–C shows that the EPS at all concentrations (0.25, 0.5, 1.0, 2.0, and 3.0 mg/mL) exhibited clear scavenging activities against the radicals in a concentration-dependent manner, and the corresponding range of scavenging activity was 15.07 to 75.10% on hydroxyl radical, 3.42 to

62.71% on superoxide anion, and 4.91 to 35.11% on DPPH. A slight increase in scavenging capacity with the concentration of EPS was observed at EPS concentrations higher than 2.0 mg/mL. This might be due to the decreased solubility of EPS, as indicated by the laser light scattering test showing the increased particle size of polysaccharide at the concentration higher than 2.0 mg/mL (data not shown). The half maximal inhibitory concentration values of EPS on hydroxyl radicals, superoxide anion, and DPPH were 1.22, 1.54, and 0.63 mg/mL, corresponding to the activity of ascorbic acid at 0.47, 0.71, and 0.61 mg/mL, respectively. The half maximal inhibitory concentration values concentration of EPS on the ferrous ion was 1.07 mg/mL, with that of EDTA at 0.29 mg/mL. Their maximal scavenging rates were reached at the concentration of 3.0 mg/mL, being 75.10% for hydroxyl radical and 62.71% for superoxide anion. These maximal scavenging rates accounted for 88.23 and 76.54% of the capacity of the positive control (ascorbic acid), respectively. Among ROS, hydroxyl and superoxide radicals were strong oxidative radicals that could severely injure biomolecules, leading to aging, cancer, and declined physiological function (Li et al., 2014). Superoxide radicals were precursors to lipid peroxidants that reacted with cell membrane to cause tissue damage (Li et al., 2014). Figure 1B indicates a relatively low scavenging capacity of EPS against DPPH (35.11%), which was 43.75% of that for ascorbic acid. A similar low scavenging capacity of microbial EPS against DPPH was reported earlier (Li and Shah, 2014; Ye et al., 2016); however, a mixture of the EPS from different microorganisms demonstrated better DPPH-scavenging capacity (Chen et al., 2015b) and the reason was unclear.

Ferrous ion has been shown to be a generator of hydroxyl radicals via Haber-Weiss reaction $\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \cdot\text{OH}$ (Panat et al., 2016). Chelating ferrous ion was considered as a secondary mechanism to estimate the antioxidant activity of compound, besides directly scavenging hydroxyl radicals (Santos et al., 2017). Figure 1D shows that the ferrous chelating rate of the EPS from *L. plantarum* YW11 increased along with the concentration, and the highest chelating rate (41.09%) achieved was close to half of the capacity of EDTA (86.25%). The metal ion-chelating ability of LAB has been widely reported; some strains were studied for application in the removal of heavy metals in a polluted environment (Yin et al., 2016). However, the ion-chelating ability of EPS produced by LAB is not well studied. Some EPS exhibited no metal ion-chelating capacity (Wang et al., 2015b), whereas some showed a strong chelating effect (Polak-Berecka et al., 2014); this difference needs to be further studied.

Antioxidant Activity of the EPS Produced by FM in the Aging Mice Model

An aging mouse model was applied to further analyze the antioxidant activity of EPS, which was induced by a subcutaneous injection of 500 mg/kg per day of 5% D-galactose solution. The effectiveness of this model was proved in the previous reports (Prakash and Kumar, 2013; Yu et al., 2016). As shown in Table 1, the mice with oxidative stress were fed LD (20 mg/kg per day) and HD (50 mg/kg per day) of EPS produced by *L. plantarum* YW11, and the FM with *L. plantarum* YW11 as therapy groups. The mice fed VC (20 mg/kg per day) were used as a positive control, and the D-galactose-treated mice as a negative control.

As shown in Figure 2, the mice treated only with D-galactose showed a severe increase in MDA activity and a decrease in GSH-Px, SOD, CAT, and T-AOC activities in serum compared with the normal group, indicating an oxidation in mice (Ho et al., 2003). The MDA level in the serum of D-galactose-treated mice was 11.69 mmol/mL ($P < 0.05$). The MDA levels of HD group (5.89 mmol/mL; $P < 0.05$) and FM group (6.62 mmol/mL; $P < 0.05$) decreased near that of VC group (5.66 mmol/mL; $P < 0.05$) and normal group mice (5.24 mmol/mL; $P < 0.05$), indicating an effective blocking of lipid oxidation in serum by EPS and fermented milk with *L. plantarum* YW11. Similarly, the EPS from *Bifidobacteria* could lower the MDA level of mice to a normal level (Xu et al., 2011).

Figure 1. Scavenging capacities of the exopolysaccharide (EPS) produced by *Lactobacillus plantarum* YW11 on hydroxyl radicals (A), 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radicals (B), and superoxide anion (C), as well as Fe^{2+} chelating ability of EPS (D). Different letters (a-d; v-z) denote significantly different values ($P < 0.05$) in scavenging or chelating rates within a treatment at different EPS concentrations and between treatments at a given EPS concentration. Points represent the mean values of scavenging or chelating rate, and the error bars represent the SD.

Figure 2. Effects of different treatments on the malondialdehyde (MDA) level, glutathione peroxidase (GSH-Px) activity, superoxide dismutase (SOD) and catalase (CAT) levels, and total antioxidant capacity (T-AOC) in the serum of mice, which were determined using ELISA. The experimental animal groups used were normal, aging (treated with D-galactose to induce oxidative stress), vitamin C (VC; treated with ascorbic acid) low dose [LD; treated with 20 mg/kg per day of exopolysaccharide (EPS) produced by *Lactobacillus plantarum* YW11], high dose (HD; treated with a 50 mg/kg per day of EPS), and fermented milk (FM; treated with milk fermented with *Lactobacillus plantarum* YW11). An asterisk (*) represents a significant difference from that in the aging group ($P < 0.05$). The box plot represents median, inter-quartile range, minimum and maximum values of data.

Among the 3 treatment groups (LD, HD, and FM), HD group had the highest serum activities of GSH-Px, SOD, CAT, and T-AOC ($P < 0.05$). Compared with the D-galactose-treated mice, the activities of these enzymes in the HD group increased by 21.55, 33.14, 61.09, and 38.18%, respectively; the CAT activities were higher than those in the VC group ($P < 0.05$). This indicated that the EPS produced by *L. plantarum*

YW11 attenuated the decrease in the antioxidant enzymes in blood serum, stimulating a consequent recovery toward normality.

Although the HD of EPS from *L. plantarum* YW11 significantly influenced the level of antioxidant enzymes in vivo, MDA, GSH-Px, SOD, and CAT activities of the LD group were not significantly different from those of aging mice ($P > 0.05$). The enzyme activities (GSH-

Px, SOD, CAT, and T-AOC) in mice serum treated by FM containing 1/10 amount of EPS (90–100 mg/L), corresponding to the LD group, increased by 7.65, 20.80, 43.31, and 31.05% ($P < 0.05$), respectively. The insignificant effect of low-dose EPS in vivo indicated a dose-dependent antioxidant activity of EPS. The controversial results of the LD and FM groups might be due to the presence of other antioxidant components in fermented milk. Many antioxidant ingredients were reported in fermented milk, including peptides (Sah et al., 2014), fatty acids (Kusche et al., 2015), riboflavin (Thakur et al., 2016), microorganisms (Gan et al., 2014), and enzymes (Kumar et al., 2010), which could increase the antioxidant activity of fermented milk by 175 to 358% (Gagnon et al., 2015).

The antioxidant activities of *L. plantarum* strains, both in vitro and in vivo (Amaretti et al., 2013; Kumar et al., 2012; Li et al., 2012), and the in vitro antioxidant activity of their EPS have been reported (Arun et al., 2017; Zhang et al., 2013), but the in vivo antioxidant activities of these EPS and their effective doses are still not clear. In our study, the EPS produced by *L. plantarum* YW11 was found to effectively improve the index of oxidative status in an aging mouse model in a dose-dependent manner. Other EPS isolated from kefir was shown to be fermented by the gut microbiota and thus influence its structure (Serafini et al., 2014). As the gut microbiota has been proven to be correlated with the oxidative status of the host (Jones et al., 2012; Clark et al., 2015), the influence of EPS on the gut microbiota and consequent effect on the oxidative status of aging mice were studied.

Gut Microbiota Changes in Mice Treated with EPS and Fermented Milk

The gut microbiota changes in mice in response to different treatments were studied using a high-throughput DNA sequencing technique (Wang et al., 2012).

Diversity Change Analysis. A total of 171,587 qualified pyrosequencing reads were obtained from 6 group of samples. After eliminating sequences without contiguous neighbors (bacteria with a very similar 16S sequence to the submitted sequence, as defined by the default criteria in the database of Greengenes), reads were delineated into 445 OTU on the 97% similarity level. Values of range (0.977–0.996; $P < 0.01$) and median (0.982; $P < 0.01$) coverage of OTU showed that the sequencing depth was sufficient. The diversity change in intestinal microbiota of different groups was examined using these OTU.

Observed species index, Shannon-Wiener index (SWI), and Simpson index (Strong, 2016) were calculated to measure the α diversity. As shown in Figure

3A, the observed species index (285 OTU) of the aging mice group was the lowest among the 6 groups ($P < 0.05$); it lost 18.57% phylotypes of gut microbiota compared with the normal group (350 OTU). This indicated that the oxidative status of D-galactose-induced aging mice changed the phylotype richness of gut microbiota. The HD group (337 OTU) showed the least difference in phylotype richness ($P < 0.05$), suggesting a better recovery effect of HD EPS treatment on the phylotype richness of intestinal microbiota in aging mice. Observed species indexes of the VC, LD, and FM groups were 320, 292, and 293 OTU, respectively, with no significant difference ($P > 0.05$) between the LD and FM groups.

The SWI and Simpson index were also assessed to investigate the phylotypes evenness of gut microbiota (Brackney et al., 2015). The evenness in the gut microbiota of the HD group (SWI = 4.27; $P < 0.05$) was the highest, and that of the aging group (SWI = 2.86; $P < 0.05$) was the poorest. The evenness of the normal group (SWI = 3.62), VC (SWI = 3.73), LD (SWI = 3.86), and FM (SWI = 3.85) groups was also better than that of the aging group. These results indicated that, although fermented milk and low-dose EPS treatment exerted a weak effect on recovering the phylotype richness of aging mice compared with HD and VC treatments ($P < 0.05$), they could affect the diversity of microbiota by changing the phylotype evenness.

For direct perception of the difference between each treatment on microbiota, reduced analyzing dimension of unweighted UniFrac data were employed using PCoA (Dominguez-Bello et al., 2010). The horizontal axis (PCoA1) in Figure 3B explains 32.31% of total variance, and the vertical axis (PCoA2) explains 23.07%. The distance of symbols represents the degree of difference between groups they stand for. The symbol of the aging mice group shows the farthest distance to the symbol of the normal group along the PCoA2 axis. The symbols of EPS (LD and HD groups) are located in the opposite direction, indicating that the influence of EPS on microbiota was opposite that of D-galactose (aging mice group). The distances between symbols representing HD and VC are shorter than others to the symbol of normal mice, showing that the recovery effects of the 2 groups on microbiota diversity were better than those of other groups. The symbol of the FM group has the longest distance to the symbols of the other groups, indicating that the milk fermented by *L. plantarum* YW11 influenced gut microbiota in a unique pattern. The analysis of unweighted UniFrac diversity distance (Figure 3C) was confirmed by results of PCoA.

Current conclusions about the relationship between gut microbiota diversity and oxidative status in aging hosts are based on specific species of strains, such as

Figure 3. Diversity changes in microbiota. (A) Observed-species index, Shannon–Wiener index, and Simpson index, with values expressed as means $\pm$ SD. An asterisk (*) represents a significant difference compared with D-galactose-treated mice ($P < 0.05$). Principal coordinate analysis (PCoA; B) of the diversity distance and unweighted UniFrac diversity distance heatmap (C) were used to exhibit β diversity distance distribution and the difference between groups of normal and aging mice, and mice treated with ascorbic acid, a low dose of exopolysaccharide (EPS) produced by *Lactobacillus plantarum* YW11, a high dose of EPS, and milk fermented by *L. plantarum* YW11. Each point represents the mean principal component scores of all rats in a group. Samples in heatmap (C) are also clustered according to their similarity. Color version available online.

Helicobacter pylori with lipid peroxidation (Davi et al., 2005), or interactions of microbiota with the aging process in insect models (Wong et al., 2011; Cabreiro and Gems, 2013). In our study, a D-galactose-induced aging mouse model was employed and treatments with ascorbic acid, EPS, and fermented milk were found to affect the diversity recovery, abundance, and evenness of gut microbiota to different extents. Microbiota variations in response to different treatments were analyzed at the family and genus levels to further study the influence of different treatments on gut microbiota.

Key Phylotypes of the Gut Microbiota Responding to EPS and Other Treatments. A redundancy analysis (Figure 4) was employed to analyze the pyrosequencing data of these samples for identifying key phylotypes of the gut microbiota responding to different treatments (D-galactose, VC, EPS, and FM). Overall, 232 key OTU were identified with at least 10% of their variability explained by the horizontal axis. The corresponding families of the OTU (Figure 5 and Supplemental Table S1; <https://doi.org/10.3168/jds.2016-12480>) were mainly *Lachnospiraceae* (51 OTU), *S24-7* (39 OTU), *Ruminococcaceae* (34 OTU), *Rikenellaceae* (10 OTU), *Desulfovibrionaceae* (7 OTU), *Bacteroidaceae* (6 OTU), *Paraprevotellaceae* (4 OTU), *Prevotellaceae* (4 OTU), *Lactobacillaceae* (4 OTU), *Helicobacteraceae* (3 OTU), *Anaeroplasmataceae* (3 OTU), *Odoribacteraceae* (3 OTU), *Porphyromonadaceae* (3 OTU), *Alcaligenaceae* (3 OTU), *Clostridiaceae* (3 OTU), and *Erysipelotrichaceae* (3 OTU). A total of 152 key OTU were decreased or eliminated in mice treated with D-galactose compared with the normal group. After the aging mice were treated with VC, LD EPS, HD EPS, and FM, the key phylotypes increased by 19, 16, 46, and 29 OTU, respectively.

The horizontal axis in Figure 4 explains 29.99% of the total variability, and the vertical axis explains 19.40% of the total variability. The effects of all treatments on the gut microbiota were significant, as tested by the Monte Carlo permutation procedure ($P = 0.016$). The symbols representing the HD and normal groups are located in the first quadrant, whereas the symbols of the LD and aging groups of mice are in the opposite quadrant. Key OTU decreased in aging mice were recovered by 59.87% after the HD EPS treatment. The samples treated with VC and FM were distributed in the second and fourth quadrants, respectively, suggesting that their effect on the aging mice was different and also differed from that of normal mice.

The analysis at the genus level showed that OTU belonging to *Flexispira* were significantly enriched in the D-galactose group (79.99%; $P < 0.0001$), whereas they were significantly lowered in the LD (8.77%), HD (2.66%), and FM (13.14%) groups. *Flexispira* has been

Figure 4. Distance triplot of the redundancy analysis of gut microbiota. Environmental variables [D-galactose (gal), ascorbic acid, exopolysaccharide (EPS), and fermented milk] are indicated by gray (red) thick arrows. Samples are represented by filled circles, including normal group, aging group, and groups treated by ascorbic acid, a low dose of EPS, a high dose of EPS, and milk fermented by *Lactobacillus plantarum* YW11. Key operational taxonomic units (OTU, 232 pieces) identified by the analysis are represented by thin black arrows. OTU better fitting to species are labeled with genus or family name. S24-7 and RF32 are unidentified families with unculturable characteristics and less similarity of their 16S rDNA sequences to any known families. *P*-value (0.016) was obtained by Monte Carlo permutation procedure.

reported to be recurrent in humans with inflammatory diseases (Gerrard et al., 2001), chronic diarrhea (Archer et al., 1988), gastrointestinal inflammation, or ulcer (Graham et al., 2007). The significant increase in richness of this genus in aging mice suggested that it might be related to the oxidative status of aging mice. The OTU belonging to *Blautia* (36.5-fold, $P = 0.022$) and *Prevotella* (12.87-fold, $P = 0.017$) were markedly enriched by VC and HD of EPS; OTU belonging to *Butyrivibrio* (9.5-fold, $P = 0.033$) significantly enriched by HD of EPS and FM; OTU belonging to *Allobaculum* enriched by FM (Figure 4 and Supplemental Table S2;

<https://doi.org/10.3168/jds.2016-12480>). Other OTU with relative abundance enriched by the HD of EPS, compared with D-galactose group, included AF12 (1 OTU), *Anaeroplasm* (1 OTU), *Bacteroides* (6 OTU), *Bilophila* (1 OTU), *Clostridium* (4 OTU), *Dehalobacterium* (2 OTU), *Desulfovibrio* (2 OTU), *Mucispirillum* (1 OTU), *Odoribacter* (3 OTU), *Prevotella* (7 OTU), *Rikenella* (2 OTU), *Ruminococcus* (9 OTU), *Lactobacillus* (4 OTU), and *Oscillospira* (19 OTU). Previously, *Bacteroides*, *Ruminococcus*, and *Prevotella* were reported to be the dominant genera in the mammalian intestinal tract. Arumugam et al. (2011) classified the gut

Figure 5. Heat map of 232 key operational taxonomic units (OTU) identified by redundancy analysis corresponding to different treatments. The relative abundance of OTU was taken after normalization and log-transformation; OTU were arranged by their sequence order in the neighbor-joining tree. The genus and family of the OTU are listed on the right. S24-7 and AF12 are the unidentified family and genus, respectively, with unculturable characteristics and less similarity of their 16S rDNA sequences to any known families or genera. Color version available online.

microbiota of Europeans into 3 enterotypes based on the relative abundance of these 3 genera. Whether such a classification of gut microbiota is suitable for mice needs further study, although the abundance of these 3 genera increased in aging mice treated with HD EPS in the current study. *Oscillospira* and *Bilophila* belong to *Firmicutes*. *Oscillospira* is a dominant genus in the gut microbiota of ruminants; it is also present in the human intestinal tract. A previous report indicated that *Oscillospira* strains specialized in fermenting complex materials of low digestibility (Tims et al., 2013). This characteristic of *Oscillospira* explained why its abundance selectively increased with EPS treatment in our study. *Bilophila* is considered to be a disease-associated genus, with a causal relationship to colorectal carcinogenesis (Andersen and Vogel, 2014). The abundance of *Bilophila* in the gut of mice fed FM changed insignificantly ($P = 0.67$, Supplemental Table S2), but increased significantly ($P = 0.0061$, Supplemental Table S2) in mice fed with HD EPS in the current study; how EPS specifically increased the growth of *Bilophila* in mice gut was unclear. The EPS produced by some bacteria might be used as the energy source for their growth (Pham et al., 2000). The abundance of *Lactobacillus* in HD group was 86.44% that of the normal group, and higher than that of the FM group (74.57%) in which mice were fed fermented milk containing *L. plantarum* YW11. This indicated that EPS might function as a prebiotic to effectively promote the growth of lactobacilli, increasing health-benefiting microorganisms in the gut microbiota.

Microbiota modulatory function was considered as one of the most important mechanisms for probiotics exerting their health-benefiting effects on the host (Kristensen et al., 2016). Exopolysaccharides, as a key metabolite of some probiotics, might play an important role in modulating gut microbiota, as some of them were found to be used by other probiotics (*Bifidobacterium*; Hongpattarakere et al., 2012) and inhibit the growth of nonprobiotic bacteria (*Enterobacteriaceae*) in vitro (Das et al., 2014). In our study, the EPS produced by *L. plantarum* YW11 was shown to modulate the microbiota in vivo by not only significantly changing the abundance and phylotypes of the whole microbiota, but also selectively changing the abundance of some key genera related to the oxidative status of host intestines. These findings also explained the consistent effect of EPS on the diversity of microbiota and oxidative status of the host. The effect of the EPS and fermented milk with *L. plantarum* YW11 on the changes in key metabolites of gut microbiota closely correlated with the oxidative status and health of mice intestinal tract was further investigated.

Concentrations of NO_x and SCFA in Feces

Excessive NO represents a factor of oxidative injuries to many visceral organs (Beckman et al., 1990); it is unstable and readily converted into NO_x (Soleimani et al., 2016), which makes NO_x a useful index to evaluate the oxidative status of intestinal environment (Coskun Cevher et al., 2015). The effect of different treatments on the concentration of NO_x in the feces is shown in Figure 6A. Among all the treatment groups, the D-galactose-treated group had the highest concentration of NO_x (194.20 $\mu\text{mol/g}$). The LD (45.31 $\mu\text{mol/g}$), HD (19.18 $\mu\text{mol/g}$) and FM (53.29 $\mu\text{mol/g}$) treatments reduced NO_x concentration compared with the normal (55.10 $\mu\text{mol/g}$) and VC groups (62.42 $\mu\text{mol/g}$). This significant reducing effect of the LD, HD, and FM groups might be due to the effective elimination of *Flexispira* strains, a reduction of 11.4, 37.5, and 7.6 fold that in the D-galactose-treated group, respectively. *Flexispira* was found to be closely relate to the members of the genus *Helicobacter*, which correlated with many types of hepatic disease (Fox et al., 2009). The strains in this family could express inducible nitric oxide synthase to cause the excessive accumulation of NO in organs, resulting in abdominal viscus injuries (Zhang et al., 2015).

The SCFA-producing genera of *Blautia*, *Butyricicoccus*, and *Allobaculum* also increased remarkably in the groups treated with VC, HD, and FM. The concentrations of acetic, propionic, and butyric acids in the feces were examined. The concentrations of acetic and butyric acids increased significantly ($P < 0.05$) in the VC (2.30 and 15.15 mg/g), HD (2.91 and 12.92 mg/g), and FM (2.43 and 14.18 mg/g) groups compared with D-galactose-treated group. The difference in propionic acid content was not significant between these groups ($P > 0.05$). The SCFA were thought to be the main energy source for intestinal epithelial cells and played a key role in the fortification of gut barrier through activating cell differentiation or participating in tight junction assembly (Cani et al., 2009; Peng et al., 2009). The in vivo gut barrier and protecting effects of plant polysaccharides and yogurt were reported earlier (Huang et al., 2008; Irvine et al., 2010). The change in fecal SCFA in the different treatment groups suggested that the protective function of an HD of EPS produced by *L. plantarum* YW11 and its fermented milk could be mediated by enriching the SCFA-producing strains in gut microbiota.

The effect of essential foods, including animal protein and fat diets and vegetarian diets, on the levels of health-related microbiota metabolites has been extensively studied. Dietary fiber in vegetarian diets proved

to be most effective to promote the SCFA production of microbiota (Vipperla and O'Keefe, 2016). This was consistent with the results obtained in our study in

the favorable effect of EPS on the SCFA formation in the mice gut. Dietary fiber could also downregulate NO production in trinitrobenzenesulfonic acid-induced colitis (Rodríguez-Cabezas et al., 2002). However, the animal source food was found to increase the abundance of *Leishmania*, associated with the induction of NO synthases (Lima-Junior et al., 2013). The high-fat diet lowered the effectiveness of NO synthase inhibitor (nitro-L-arginine; Bhattarai et al., 2016); however, a direct evidence of dietary effect on the level of NO in the intestinal tract is still scarce. This novel study reported the regulatory effect of EPS produced by *L. plantarum*, similar to that of dietary fiber, on the NO and SCFA levels in aging mice intestines, thereby facilitating the recovery of intestinal oxidative status. The correlation of gut microbiota change with other phenotypes (MDA, GSH-Px, SOD, CAT, and T-AOC) more directly reflecting the oxidative status of aging mice was analyzed.

Prediction of Metabolic Phenotypes in a Host via PLS Regression Model

The PLS regression models were applied to assess the possible associations between the microbiota variations and antioxidant phenotype change in aging mice, using the 232 key OTU determined by previous RDA analysis. As shown by the goodness of prediction (Q^2) in Table 2, PLS-regression models performed well with CAT ($Q^2 = 0.49$), T-AOC ($Q^2 = 0.45$), NO_x ($Q^2 = 0.67$), and SCFA ($Q^2 = 0.55$), indicating that the identified key OTU had a good correlation with the antioxidant phenotype of mice. The prediction quality of the models was also validated by the result of Pearson correlation analysis of the predicted and observed values, especially for CAT ($R = -0.67$), T-AOC ($R = -0.72$), and SCFA ($R = -0.81$) activities. Previously, the oxidative-stress metabolic diseases and hypofunction, including colon cancer (Sears and Garrett, 2014), cardiovascular disease (Siti et al., 2015), diabetes (Karunakaran and Park, 2013), obesity (Nistala et al., 2014), and aging (Biagi et al., 2013), were found to have different extent of correlation with gut microbiota (Sanz et al., 2015). However, the conclusions based on human models are controversial mainly due to the long aging process in humans and complex uncertainties during the experiment. A reproducible aging mouse model was employed in our study, and a good correlation of gut microbiota with the oxidative status of D-galactose-induced aging mice was obtained. The mouse was considered as an irreplaceable animal model due to its benefits in the study of the response of gut microbiota to perturbations in a controlled experimental setup testifying causable

Figure 6. Effect of different treatments on the concentrations of nitrogen oxides (NO_x , A) and short-chain fatty acids (SCFA, B) in the feces of aging mouse model. An asterisk (*) represents a statistically significant difference ($P < 0.05$) comparing the individual group with the aging group assessed by the 1-way ANOVA test. EPS = exopolysaccharide. Bar heights represent the mean values of concentration of SCFA or NO_x ; error bars represent the value of SD.

Table 2. Predicted correlation of mice phenotypes with the key OTU using PLS regression model^{1,2}

Item	Q ²	RMSECV	r	P-value
MDA	0.23	2.91	−0.75	0.0086
GSH-Px	0.18	43.71	0.22	0.0361
SOD	0.14	25.13	−0.15	0.0031
CAT	0.49	3.37	−0.67	0.0148
T-AOC	0.45	3.72	−0.72	0.0107
NO _x	0.67	7.81	−0.66	0.0131
SCFA ³	0.55	3.72	0.81	0.0247

¹Q² = quality of prediction regressed by leave-one-out cross-validated partial least square regression (PLS); RMSECV = root mean squared error of cross validation; r and P-values are of predicted and observed values.

²Key OUT = key operational taxonomic units identified by redundancy analysis; MDA = malondialdehyde; GSH-Px = glutathione peroxidase; SOD = superoxide dismutase; CAT = catalase; T-AOC = total antioxidant capacity; NO_x = nitrogen oxides; SCFA = short-chain fatty acids.

³Total content of acetic, propionic, and butyric acids.

relations (Smits et al., 2013; Brahe et al., 2016; Mai et al., 2016). The results obtained from last 10-yr of studies with an obesity mice model on the relationship of gut microbiota with inflammatory bowel disease and obesity proved effective in humans (Nguyen et al., 2015).

CONCLUSIONS

The EPS produced by *L. plantarum* YW11 had a strong antioxidant activity in vitro and in vivo. A high dose of EPS (50 mg/kg per day) effectively relieved the oxidative stress in aging mice, with increased serum levels of GSH-Px, SOD, CAT, and T-AOC, but decreased MDA level. The EPS could effectively recover the diversity and phylotypes decreased or eliminated in the untreated aging mouse model to similar levels as those in the normal mice. The EPS also selectively decreased the abundance of *Flexispira* and increased the abundance of *Blautia* and *Butyricicoccus*, and correspondingly decreased the fecal content of NO_x and increased the content of SCFA, thereby improving the oxidative and health conditions of the host intestinal tract. Variation of key phylotypes in gut microbiota induced by different doses of EPS treatment had strong correlation with oxidative phenotypes of mice. The fermented milk with *L. plantarum* YW11 containing EPS also showed favorable antioxidant and gut microbiota-regulating activities. The results obtained in this study showed that EPS produced by *L. plantarum* YW11 could modulate gut microbiota and improve the antioxidant status of the host, contributing to further understanding of the functional mechanism of probiotics and probiotic foods.

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