A novel strain of *Lactobacillus mucosae* isolated from a Gaotian villager improves in vitro and in vivo antioxidant as well as biological properties in D-galactose-induced aging mice

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ABSTRACT

Twelve isolates isolated from the gastrointestinal tracts of Gaotian villagers in China, who had a lifespan of 92 yr, were examined for their antioxidants using free radical scavenging activity and 2,2-diphenyl-1-picrylhydrazyl. Three strains (i.e., *Lactobacillus mucosae* LMU1001, and *Lactobacillus plantarum* LPL0902 and LPL0302) were selected as candidates to prepare yogurt for testing their antioxidants in a model of D-galactose-induced aging mice, with vitamin C as a positive control. The results showed that *L. mucosae* LMU1001 was the best strain, which had similar in vivo antioxidant activity as vitamin C. A significant increase was found in the activities of glutathione peroxidase in serum and total superoxide dismutase in the liver, and a decrease in the level of malondialdehyde in serum. Regarding mRNA expression level detected quantitatively by real-time PCR, we observed that *L. mucosae* LMU1001 significantly upregulated antioxidant genes (i.e., *MT1A* and *MT1M* in HT-29 and Caco-2) and those genes (i.e., *MT1*, *MT2*, *Gpx1*, and *Gpx2*) in the intestinal tract of the model mice. Hence, this strain could be considered as a potential probiotic lactic acid bacterium for improving antioxidant levels in functional foods.

Key words: antioxidant activity, 2,2-diphenyl-1-picrylhydrazyl, hydroxyl radical, reducing power, D-galactose-induced aging mice

INTRODUCTION

It has been suggested that longevity may be related to many factors, such as heredity (Deelen et al., 2014), environment (Prather et al., 2015), and constitution

of gut flora (Biagi et al., 2015), or even some special bacterial species. In the last decade, some researchers focused on the global microbiota analysis of people with longevity (Zhao et al., 2011). Probiotics including *Lactobacillus* and *Bifidobacterium* have attracted much attention due to their role in health promotion. Healthy people, especially with longevity, were the main target for isolating probiotics all over the world (Woo et al., 2014). *Bifidobacterium longum* ssp. *longum* BBMN68 isolated from healthy centenarians in Bama, China, was reported to enhance immune function in BALB/c mice (Yang et al., 2009). Similar to Bama and other areas of people with longevity, the Gaotian village, located in Liuyang city of Hunan province in the middle-south of China, boasts people with a lifespan of up to 92 yr with no history of chronic diseases. However, the microbial community of those people has not been studied so far and the reasons for their longevity are still unknown. Therefore, a systematic study of the microflora of the Gaotian villager is of utmost importance to understand a relationship between the constituents of microflora and health status.

Recently antioxidant potential of lactic acid bacteria (LAB) and their components (e.g., exopolysaccharides and proteins) was frequently reported and even regarded as one of the standard for evaluation of probiotic functions (Li et al., 2014a; Li and Shah, 2014). Evaluation of antioxidant properties includes in vitro oxidation reaction system and in vivo animal testing; the former covers a series of protocols for scavenging free radicals, chelating metal ions, and inhibiting lipid peroxidation (Makarova et al., 2006), whereas the latter covers monitoring change in the antioxidant enzyme system [i.e., glutathione peroxidase, superoxide dismutase, and malondialdehyde (MDA)] content of host (LeBlanc et al., 2011). Zhang et al. (2010) used a hyperlipidemic model rat, which was induced by a high-fat diet, to test antioxidant properties, and demonstrated that

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Lactobacillus casei Zhang alleviated oxidative stress by reducing lipid peroxidation, and improved lipid metabolism both in blood and liver of the rat. Kanno et al. (2012) reported that *Lactobacillus plantarum* 7FM10 from the traditional Japanese food narezushi exhibited 2,2-diphenyl-1-picrylhydrazyl (DPPH) and superoxide radical scavenging capabilities (Kanno et al., 2012).

To date, several reports pertaining to antioxidant evaluation of LAB have been published; however, a systematic evaluation of the antioxidant mechanism from cell lines to model mice, from metabolic substances to the gene expression level, has not been reported yet.

In our previous study (Yu et al., 2015), the gut microbial community of long-living elderly residents of a Gaotian village was analyzed systematically using 16S high-throughput sequencing, and 12 of 82 isolates were screened and studied for their probiotic and biological characteristics (data not shown). To obtain an overview of the functional properties of the potential strains, in this study, we first investigated their antioxidant capability. Second, we investigated the effects of yogurt on the physiological and biochemical index of serum and organ by using D-galactose (D-Gal)-induced aging mice. Subsequently, we examined the antioxidant mechanisms of selective LAB strains in cell lines of HT-29 and Caco-2. Some important host oxidant genes (i.e., *MT1*, *MT2*) and enzyme (i.e., *SOD*, *GSH-PX*) were quantitatively detected with the aid of biochemical analysis and real-time PCR technique.

MATERIALS AND METHODS

Bacteria Strains and Culture

Twelve strains isolated from the intestinal tract of long-living elderly residents of a Gaotian village of Liuyang City, Hunan, China, including *L. plantarum* LPL0105, LPL0302, and LPL0902, *L. fermentum* LFE0608, LFEP3, and LFEP6, *L. salivarius* LSAP2 and LSAP5, *L. mucosae* LUM1001, *Enterococcus hirae* P1, and *Enterococcus faecalis* P4, were used in this study. All the strains were cultured anaerobically in de Man, Rogosa, Sharpe broth (MRS; Beijing Solarbio Science and Technology Co. Ltd., Beijing, China) medium at 37°C for 18 h.

Preparation of Cells, Supernatants of Different Strains, and Yogurt

Cells of 12 strains were harvested by centrifugation (Thermo Fisher Sorvall ST 16R, Schwerte, Germany) at 4°C, $8,000 \times g$, for 10 min after culturing anaerobically in 50 mL of MRS broth (1%, vol/vol) at 37°C for 18 h.

The supernatants were decanted. The cell pellets were then washed in triplicates in PBS and resuspended in 50 mL of PBS. For preparation of yogurt using the 12 strains, 100 mL of skim milk was inoculated with 1% of overnight culture of each strain and then incubated anaerobically at 37°C for 24 h. Both cells and supernatants of 12 strains, and 12 batches of yogurt, were stored at 4°C for use.

Antioxidant Activity by Scavenging DPPH Free Radical

The DPPH free radical scavenging activity was determined according to the method of Li and Shah (2014). Briefly, 0.2 mM DPPH (Sigma Chemical Co.) solution in 100 mL of ethanol was prepared, 1.0 mL of each sample and ethanol as control were mixed with 4.0 mL of ethanolic DPPH solution, respectively, and incubated in the dark for 30 min. The blank was 4.0 mL of ethanol mixed with 1.0 mL of MRS or PBS. The absorbance of supernatant was measured at 517 nm after centrifugation at room temperature (23°C) at $4,500 \times g$ for 10 min. The scavenging of DPPH radical was calculated as follows: scavenging ability (%) = $100 - (A_{\text{sample}} - A_{\text{blank}})/A_{\text{control}} \times 100$.

Antioxidant Activity by Scavenging Hydroxyl Radical

The scavenging activity of hydroxyl radical was determined by a Fenton reaction method (Ferreira et al., 2007; Arasu et al., 2014). Briefly, the reaction mixture contained 1.0 mL of PBS (0.02 mM, pH 7.4), 0.5 mL of 1,10-phenanthroline monohydrate (2.5 mM), 0.5 mL of FeSO₄ (2.5 mM), and 0.5 mL of H₂O₂ (20 mM). The mixture was mixed with 0.5 mL of each sample, and maintained at 37°C for 1 h. The absorbance of the mixture was measured at 536 nm and the equation of hydroxyl radical scavenging activity is expressed as follows: scavenging ability (%) = $[(A_s - A_o)/(A - A_o)] \times 100$, where A_s is the absorbance of tested sample, A_o is the absorbance of the control, and the A is the absorbance without sample and H₂O₂.

Reducing Power

Detection of reducing power was carried out according to the method of Marazza et al. (2012), with minor modifications. Briefly, an aliquot of 0.5 mL of the sample was mixed with 0.5 mL of PBS (0.2 M, pH 6.6) and 0.5 mL of 0.1% potassium ferric cyanide and mixed. The mixed solution was added with the same volume of 10% trichloroacetic acid to stop the reaction after incubation at 50°C for 20 min. This was followed

by mixing 0.1 mL of ferric chloride (0.1%) with the supernatants, which was obtained after centrifugation at 4°C, $3,000 \times g$ for 10 min. After reaction for 10 min, the absorbance of the solution measured at 700 nm. Higher absorbance means higher reducing power.

Animals and Animal Experiments

The protocol for the animal experiment was approved by Nanchang University Animal Ethical Committee and met all ethical requirements to conduct the experiment (approval number 0064257).

Sixty 5-wk-old BALB/c male mice were purchased from Animal Center of Nanchang University (Jiangxi, China). Mice were initially acclimatized to the laboratory conditions for 10 d, then housed at $23 \pm 1^\circ\text{C}$, 12:12 h light-dark cycle. They were divided into 6 groups (10 in each group) at random, including 5 groups that were injected subcutaneously with D-Gal at 50 mg/kg·d BW (Song et al., 1999) and a sixth group that was injected subcutaneously with equivalent physiological saline as a control. After 30 d, 3 groups were administered via gastric gavage with fermented yogurt by 3 strains, the fourth group was gavaged with skim milk as a D-Gal model group, and the fifth group was gavaged with vitamin C, which served as a positive control. The sixth group was administered via gastric gavage with an equivalent volume of skim milk and served as the negative control. The BW was recorded after narcosis with ethyl ether, and the blood (potassium EDTA collection tube) was collected from the orbital sinus by quickly removing the eyeball. The tissue of mice was weighed and recorded after dissection.

Biochemical Analysis

The serum of the mice was prepared by storing the whole blood at 4°C overnight and centrifuged (Thermo Fisher Sorvall ST 16R) at 4°C, $2,000 \times g$ for 8 min. For evaluation of antioxidant activity, the levels of glutathione peroxidase (**GSH-PX**), total superoxide dismutase (**T-SOD**), and MDA in the serum and liver were determined according to the instructions of kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). For evaluation of biochemical index, alanine transaminase (**ALT**), aspartate aminotransferase (**AST**), total bilirubin (**TBIL**), direct bilirubin (**DBIL**), creatinine (**CREA**), urea and uric acid (**UA**), blood glucose (**GLU**), triglyceride (**TG**), and total cholesterol (**T-CHOL**) were tested in the First Affiliated Hospital of Nanchang University (Ceriotti et al., 2010; Coban et al., 2014; Schulkens et al., 2014). Spleens, kidneys, hearts, and livers were weighed, and the organ index

was estimated from the ratio of organ weight to BW (organ weight/BW $\times$ 100).

Cell Lines and Tests

The human colon carcinoma cell lines HT-29 and Caco-2 were cultured on Petri dishes in Dulbecco's modified Eagle's medium (**DMEM**, Gibco, Grand Island, NY) with 10% FBS (Gibco), and propagated at 37°C under 5% CO₂. Fresh overnight cultures of tested strains, after washing triplicates with PBS, were incubated with 2 cells of Caco-2 and HT-29, respectively, in 4 mL of DMEM at 37°C under 5% CO₂ for 2 h. The ratio of cells of Caco-2 and HT-29 to that of tested strains was 1:10.

RNA Isolation and cDNA Synthesis

Total RNA was isolated according to the instructions of TaKaRa MiniBEST Universal RNA extraction kit (Takara Bio, Dalian, China). Five micrograms of total RNA was transcribed into cDNA according to the instructions of the PrimeScript 1st Strand cDNA Synthesis Kit (Takara Bio).

Real-Time PCR

The standard curve was used for quantification between the housekeeping gene and the reference gene. The PCR primers of intestine of mice are listed in Table 1, and the cell lines are listed in Table 2. Quantitative PCR amplification was performed using the ABI 7900 HT fast real-time PCR system (Applied Biosystems, Foster City, CA). The reaction of real-time PCR assays contained 10 μL of SYBR Primer EX *TaqII* (Takara Bio), 0.75 μL each of the primers with 1 μL cDNA template and 7.5 μL H₂O, and the final volume of 20 μL . The amplification was programmed to start at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The template of cDNA was run in triplicate by real-time PCR and analyzed using the $2^{-\Delta\Delta\text{Ct}}$ method based on RT-PCR data as described by Feng et al. (2010).

Statistical Analysis

All tests were carried out in triplicate for all the experiments. All data were expressed as means $\pm$ standard deviation of 3 parallel tests. The SPSS version 14.0 software was used for statistical analyses (SPSS Inc., Chicago, IL) by means of independent one-way ANOVA tests. The figures were prepared by using

Table 1. The PCR primers related to oxidative stress in real-time PCR on the intestine of mice

Gene name ¹	Forward primer (5'-3')	Reverse primer (5'-3')
<i>MT1</i>	TTCACCAGATCTCGGAATGG	GAGGTGCACTTGCAGTTCTTG
<i>MT2</i>	CCTGCAAATGCAAACAATGC	CACTTGTGCGAAGCCTCTTTG
<i>GPx1</i>	TGAGAAGTGCGAGGTGAATG	CGGGGACCAAATGATGTACT
<i>GPx2</i>	TGCCCTACCCTTATGACGAC	TCGATGTTGATGGTCTGGAA
<i>SOD</i>	CGAGGGGCATCTAGTGGAGAAG	TTAGGGCTCAGGTTTGTCCAGAA
β -Actin	AGGCCAACCAACCGCGAGAAG	GTCCAGGCGCATGATGG

¹*MT1* = metallothionein 1; *MT2* = metallothionein 2; *GPx1* = glutathione peroxidase 1; *GPx2* = glutathione peroxidase 2; *SOD* = superoxide dismutase.

GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA).

RESULTS

In Vitro Tests for Antioxidant Ability of Cells, Supernatants of Isolates, and Yogurt Made by Using Those Isolates

Twelve isolates of villagers from Gaotian, China, with a history of long lifespan were determined by 16S rDNA, and 10 of the isolates were classified as *Lactobacillus*, whereas 2 of the isolates were classified as *Enterococcus*. Further, those strains were investigated for their antioxidant ability based on the scavenging of DPPH free radicals and hydroxyl radicals, and total reducing power. Considering the contribution of antioxidant potential might come from either cells or the supernatants, a series of systematic tests was performed for evaluation of the potential application of those strains in a dairy product (e.g., yogurt). For scavenging of DPPH free radicals (Figure 1A), the behavior of cells, supernatants, and yogurt was different among 12 strains; *L. mucosae* LMU1001, *L. plantarum* LPL0902 and LPL0302, and *L. fermentum* LFE1014 demonstrated relatively higher scavenging ability in all the indices (i.e., cell counts, supernatants, and yogurt); namely, their DPPH free radical scavenging ability of cells, supernatants of isolates, and yogurt exceeded 60% (72.78, 63.28, and 88.15% vs. 69.50, 75.00, and

71.39% vs. 66.25, 64.75, and 75.56% vs. 64.50, 67.30, and 68.13%, respectively). For scavenging of hydroxyl radicals (Figure 1B), in general, supernatants demonstrated the highest scavenging activities, at 88.25%, 83.36%, and 86.28% for *L. fermentum* LFE0608, *L. mucosae* LMU1001, and *L. salivarius* LSAP2, respectively. Similar results were achieved for reducing power tests (Figure 1C); the reducing power of supernatants was the best among tests for cell counts, supernatants of isolates, and their yogurt product. The highest reducing power was from the strain of *L. mucosae* LMU1001 (optical density at 700 nm = 2.021).

Based on the comprehensive results of scavenging of DPPH free radical and hydroxyl radical, and total reducing power of 12 strains, *L. mucosae* LMU1001 and *L. plantarum* LPL0302 were selected for their enhanced antioxidant ability in yogurt and *L. plantarum* LPL0902 for their average antioxidant ability in cells, supernatants of isolates, and yogurt product made by using it. Those 3 strains were used for the following in vivo tests.

Monitoring the Antioxidant Gene Expression Level in Cell Lines of Caco-2 and HT-29 Co-incubated with LAB by RT-PCR

Upon the above finding of the antioxidant ability of *L. mucosae* LMU1001, *L. plantarum* LPL0302, and *L. plantarum* LPL0902 in vitro, to ascertain their mechanism at the molecular level, a respective co-incubation

Table 2. Oligonucleotide primer pairs used for real-time PCR on cells

Product ¹	Forward primer (5'-3')	Reverse primer (5'-3')
β -Actin	AGCGAGCATCCCCAAAGTT	GGGCACGAAGGCTCATCATT
<i>PRG1</i>	TTTCCTGGCTTTGTGACTCC	ATGCTTTTGGTCCAGCTCC
<i>GPX2</i>	CACACCATCTCTTCAGCCTAGC	CAAGCAATCTGCCTACCTTAGC
<i>SOD2</i>	TCTTCAGCCTGCACTGAAGT	ACTGAAGGTAGTAAGCGTGC
<i>BCL2</i>	GTGTTCCGCGTGATTGAAGAC	CCCCAGAGAAAGAAGAGGAGTTATAA
<i>MT1M</i>	CCGTCCGGGTGGGCCTA	GCAGCWCTTCTTGACAGGAGG
<i>MT1A</i>	TTTCCTCCCCTAAGTGTGTGG	TCGGAGATGGGAGATAAGTGG

¹*PRG1* = p53-responsive gene 1; *GPX2* = glutathione peroxidase 2; *SOD2* = superoxide dismutase 2; *BCL2* = B cell CLL/lymphoma 2; *MT1M* = metallothionein 1M; *MT1A* = metallothionein 1A.

Figure 1. The antioxidant ability of 12 isolates from Gaotian villagers with long lifespans. Scavenging effect of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals (A) and hydroxyl radicals (B); reducing power (measured as optical density at 700 nm; C). 0105 = *Lactobacillus plantarum* LPL0105; 0302 = *L. plantarum* LPL0302; 0608 = *Lactobacillus fermentum* LFE0608; 0902 = *L. plantarum* LPL0902; 1001 = *Lactobacillus mucosae* LUM1001; P1 = *Enterococcus hirae* P1; P2 = *Lactobacillus salivarius* LSAP2; P3 = *L. fermentum* LFEP3; P4 = *Enterococcus faecalis* P4; P5 = *L. salivarius* LSAP5; P6 = *L. fermentum* LFEP6. The data are presented as means $\pm$ standard deviation.

Figure 2. Gene expression of HT-29 (A) and Caco-2 (B) in mRNA level after incubation with *Lactobacillus mucosae* LMU1001, including *PRG1*, *GPX2*, *SOD2*, *MT1A*, *MT1M*, and *BCL2*. A ratio >1 indicated upregulation of gene expression, whereas a ratio <1 indicated downregulation. * $P < 0.05$ and ** $P < 0.01$: significantly different compared with negative group. The data are presented as means $\pm$ standard deviation.

of that strain with the epithelial cell lines of HT-29 and Caco-2 was designed and a series of RT-PCR assays was performed. As shown in Figure 2, the gene expression level of the independent gene (i.e., *PRG1*, *GPX2*, *MT1A*, *MT1M*, and *BCL2*) presented an increasing trend in both cell lines, of which *MT1A* was the highest, followed by *MT1M*, and both of them together with *GPX2* were highly significant ($P < 0.01$) in Caco-2. The *PRG1* and *BCL2* were upregulated by *L. mucosae* LMU1001 either in HT-29 or Caco-2.

Effect of *L. mucosae* LMU1001 and *L. plantarum* LPL0302 and LPL0902 on Physiological and Biochemical Index and In Vivo Antioxidant Potential in D-Gal-Induced Aging Mice

Based on the results of the in vitro tests of the antioxidant ability and antioxidant gene expression level in cell lines, we speculated that *L. mucosae* LMU1001 might have an anti-aging ability and the D-Gal-induced aging model mice were continuously administered with yogurt fermented by *L. mucosae* LMU1001, and *L. plantarum* LPL0302 and LPL0902 for 1 mo.

Results of BW and organ indices (Table 3) showed no differences in BW, heart, and spleen index among D-Gal model mice, LAB administered mice, and control mice ($P > 0.05$); however, the liver and kidney index of D-Gal model mice was significantly lower ($P < 0.05$) than those of the mice administered with *L. mucosae* LMU1001 (4.91 ± 0.23 , 0.96 ± 0.24) and vitamin C (4.23 ± 0.33 , 1.16 ± 0.24), respectively, unlike the other 2 *L. plantarum*. Further, as compared with the vitamin C group, the liver index of *L. mucosae* LMU1001 group was higher ($P < 0.05$), but the kidney index was not ($P > 0.05$), indicating that *L. mucosae* LMU1001 has a similar potential to reduce the harmful effect of D-Gal on the liver of mice. Subsequently, liver function reflected by level of ALT, AST, TBIL, and DBIL in the serum was monitored, and results (Figure 3) showed that the level of ALT and AST of *L. mucosae* LMU1001 decreased significantly ($P < 0.05$, $P < 0.01$, respectively), similar to the vitamin C group ($P < 0.01$), when compared with D-Gal group, whereas 2 other LAB strains presented a nonsignificant difference. On the other hand, no differences were found in the index of TBIL and DBIL among all groups ($P > 0.05$). Results of glucose and lipid metabolism of serum (Figure 3) exhibited that GLU level increased significantly in all the groups, except the normal control group, when compared with the D-Gal group. For T-CHOL, only *L. mucosae* LMU1001 and vitamin C increased significantly when compared with the D-Gal group ($P < 0.05$). For TG, only vitamin C and *L. plantarum* LPL0302 significantly decreased their level when compared with the D-Gal group ($P < 0.01$, $P < 0.05$, respectively; Figure 4C). Results of LAB on the level of CREA, UREA, and UA in serum showed no effect, except that *L. plantarum* LPL0302 lowered the UA level.

In Vivo Test of Antioxidant Ability of Yogurt Fermented by *L. mucosae* LMU1001 and *L. plantarum* LPL0302 and LPL0902 in D-Gal-Induced Aging Mice

To further confirm in vitro antioxidant ability of the 3 LAB, the level of GSH-PX, T-SOD, and MDA in the serum and liver of D-Gal-induced aging mice was monitored. As shown in Figure 5, only *L. mucosae* LMU1001 significantly increased the level of GSH-PX and lowered the MDA in the serum. When compared with the vitamin C group, this strain showed the same trend as in vivo antioxidant tests. Similar to serum tests, for the level GSH-PX, T-SOD, and MDA in the liver (Figure 6), only *L. mucosae* LMU1001 demonstrated a significant increase in potential for T-SOD. When compared with vitamin C, that strain exhibited the

Figure 3. Biochemical analysis of serum of D-galactose (D-Gal)-induced mice administered with yogurt made by using *Lactobacillus mucosae* LMU1001, and *Lactobacillus plantarum* LPL0902 and LPL0302. The negative control group (NC) was injected subcutaneously with physiological saline and administered skim milk via gastric gavage. (A) Alanine aminotransferase (ALT), (B) aspartate aminotransferase (AST), (C) total bilirubin (TBIL), and (D) direct bilirubin (DBIL). The data are presented as means $\pm$ standard deviation ($n = 10$). * $P < 0.05$ and ** $P < 0.01$: significantly different compared with D-Gal model mice.

Table 3. Effect of administered strains on BW and organ index of mice¹

Group	BW (g)		Organ index ²			
	0 wk	7 wk	Liver	Heart	Spleen	Kidney
Negative control	23.02 $\pm$ 2.01	38.62 $\pm$ 2.62	4.84 $\pm$ 0.23 ^a	0.43 $\pm$ 0.01	0.45 $\pm$ 0.17	1.26 $\pm$ 0.26
D-Gal	22.89 $\pm$ 1.68	34.54 $\pm$ 1.28	5.69 $\pm$ 0.28	0.54 $\pm$ 0.06	0.92 $\pm$ 0.16	1.32 $\pm$ 0.03
D-Gal + LPL0302	22.54 $\pm$ 2.02	35.65 $\pm$ 0.87	4.90 $\pm$ 0.82	0.46 $\pm$ 0.02	0.67 $\pm$ 0.42	1.18 $\pm$ 0.14
D-Gal + LMU1001	23.13 $\pm$ 1.78	36.10 $\pm$ 3.09	4.91 $\pm$ 0.23 ^a	0.49 $\pm$ 0.01	0.48 $\pm$ 0.27	0.96 $\pm$ 0.24 ^a
D-Gal + LPL0902	22.98 $\pm$ 2.49	35.50 $\pm$ 1.23	5.28 $\pm$ 0.98	0.45 $\pm$ 0.01	0.43 $\pm$ 0.14	1.06 $\pm$ 0.06
D-Gal + vitamin C	23.29 $\pm$ 2.75	35.25 $\pm$ 1.94	4.23 $\pm$ 0.33 ^b	0.52 $\pm$ 0.08	1.16 $\pm$ 0.24	1.16 $\pm$ 0.24

^a $P < 0.05$.

^b $P < 0.01$ compared with D-galactose (D-Gal) model mice.

¹The data presented are means $\pm$ standard deviation ($n = 10$). LMU1001 = *Lactobacillus mucosae* LMU1001, and LPL0902 and LPL0302 = *Lactobacillus plantarum* LPL0902 and LPL0302.

²Organ index = weight of organ/BW $\times$ 100.

Figure 4. Glucose and lipid metabolism reflected by serum in D-galactose (D-Gal)-induced mice fed yogurt with *Lactobacillus mucosae* LMU1001, and *Lactobacillus plantarum* LPL0902 and LPL0302. The negative control group (NC) was injected subcutaneously with physiological saline and administered skim milk via gastric gavage. (A) Blood glucose (GLU), (B) total cholesterol (T-CHOL), and (C) triglyceride (TG). The data are presented as means $\pm$ standard deviation ($n = 10$). * $P < 0.05$ and ** $P < 0.01$: significantly different compared with D-Gal model mice.

Figure 5. The level of (A) creatinine, (B) urea, and (C) uric acid in serum of D-galactose (D-Gal)-induced mice fed yogurt with *Lactobacillus mucosae* LMU1001, and *Lactobacillus plantarum* LPL0902 and LPL0302. The negative control group (NC) was injected subcutaneously with physiological saline and administered skim milk via gastric gavage. The data are presented as means $\pm$ standard deviation ($n = 10$). * $P < 0.05$ and ** $P < 0.01$: significantly different compared with D-Gal model mice.

Figure 6. Effect of strains and vitamin C on the activity of glutathione peroxidase (GSH-PX) and total superoxide dismutase (T-SOD) as well as malondialdehyde (MDA) level in serum (A–C) and liver (D–E) of D-galactose (D-Gal)-induced mice fed yogurt with *Lactobacillus mucosae* LMU1001, and *Lactobacillus plantarum* LPL0902 and LPL0302. The negative control group (NC) was injected subcutaneously with physiological saline and administered skim milk via gastric gavage. A and D = GSH-PX activity; B and E = T-SOD activity; C and F = MDA level. The data are presented as means $\pm$ standard deviation (n = 10). * P < 0.05 and ** P < 0.01; significantly different compared with D-Gal model mice.

Figure 7. Gene expression of mRNA in the intestine tract of D-galactose (D-Gal)-induced aging mice, including *MT1*, *MT2*, *GPX1*, *GPX2*, and *SOD*. A ratio greater than 1 indicated upregulation of gene expression, whereas a ratio below 1 indicated downregulation. The data are presented as means $\pm$ standard deviation. * $P < 0.05$: significantly different compared with vitamin C group.

same potential for enhancing the antioxidant ability in D-Gal-induced aging mice.

Verification of the Regulation of Antioxidant Gene Expression in the Intestine of D-Gal-Induced Aging Mice Administered Yogurt Fermented by *L. mucosae* LMU1001

Furthermore, using vitamin C as a positive control, the antioxidant gene expression level of D-Gal-induced aging mice administered with *L. mucosae* LMU1001 was detected by RT-PCR. As shown in Figure 7, all of the genes expression inducing *MT1*, *MT2*, *GPX1*, *GPX2*, and *SOD* were upregulated in the groups of *L. mucosae* LMU1001 and vitamin C, whereas those genes were downregulated in the D-Gal model mice. The levels of *MT1* and *MT2* increased as high as 8-fold. *Lactobacillus mucosae* LMU1001 and vitamin C showed a similar efficiency to improve the antioxidant activity in D-Gal-induced aging mice, except for *SOD* gene expression.

DISCUSSION

Several studies have reported probiotic functions of LAB due to their antioxidant potential (Li et al., 2014b; Persichetti et al., 2014; Dilna et al., 2015). In this study, 12 isolates of the 82 isolates from Gaotian villagers with long lifespans were tested for their in vitro and in vivo antioxidant potential based on the contribution of cell counts, supernatants, as well as yogurts made from those isolates to examine their potential application. Three of the isolates, namely, *L. mucosae* LMU1001 and *L. plantarum* LPL0302 and LPL0902, were screened out for their antioxidant abil-

ity using scavenging of DPPH free radical and hydroxyl radical or their reducing power. Based on the results, *L. mucosae* LMU1001 was found to be the best candidate. A similar organism has been isolated from humans and their biological functionalities were reported (Roos et al., 2000; London et al., 2014). Previously, *L. mucosae* were detected in feces and mucosa-associated microbiota of short bowel syndrome patients (Joly et al., 2010) and from strongly enriched mucus in a dynamic gut model (Van den Abbeele et al., 2012).

Only a few studies report a systematic in vitro and in vivo evaluation of antioxidant activity. Upon finding significantly higher antioxidant potential strains (i.e., *L. mucosae* LMU1001 and *L. plantarum* LPL0302 and LPL0902), we report that *L. mucosae* LMU1001 decreased the level of ALT, AST, T-CHOL, and Glu, with no effect on BW and CREA. Theoretically, in vivo damage of organs by free radicals is usually reflected in the abnormal expression of organ indices and biomarkers (e.g., ATL, AST, and CREA) in the serum of aged mice (Zhang et al., 2009). Alanine transaminase and AST are the most important enzymes for judging hepatic metabolism (Mera et al., 2008; Ceriotti et al., 2010). Our results demonstrated that ALT and AST decreased significantly in *L. mucosae* LMU1001 groups, indicating their protective effect on liver. To the best of our limited knowledge, no studies have reported the effect of LAB on those 3 indices in D-Gal-induced aging mice. Only plant antioxidant materials [e.g., Korean red ginseng water extract (Ramesh et al., 2012), blueberry (Coban et al., 2014), and Troxerutin (Zhang et al., 2009)] were found to have a similar improvement in D-Gal-induced mice. Remarkably, *L. mucosae* LMU1001 was reported to have similar in vivo antioxidant ability

as vitamin C as supported by regulation in the level of GSH-PX, T-SOD, and MDA in the serum.

To ascertain the up- and downregulation of antioxidant-related genes in vivo and in vitro, we selected metallothioneins, superoxide dismutase, and GSH-PX as the target. The *MT1A* and *MT1M* are functional members of the metallothionein superfamily, which are small cysteine-rich intercellular proteins, and protect cells against oxidative stress by virtue of their free-radical-scavenging capacity and their inhibitory effect on the activity of ROS-producing enzymes (Schulkens et al., 2014). The *SOD* is a ubiquitously expressed mitochondrial antioxidant enzyme that is essential for mammalian life. The *GPX2* gene, which encodes a GSH-PX, may be used to regulate the intracellular hydroperoxide level in the gastrointestinal tract (Yan and Chen, 2006). We found that *GPX2*, *MT1A*, and *MT1M* were positively upregulated by *L. mucosae* LMU1001 either in cell lines of HT-29 and Caco-2 or D-Gal-induced model mice. Our results indicated that *L. mucosae* LMU1001 might be a potential strain for enhancing their antioxidant ability for health promotion. Our results indicated that *L. mucosae* LMU1001 might be associated with cell differentiation and the inhibition of tumors.

CONCLUSIONS

Lactobacillus mucosae LMU1001, originally isolated from a Gaotian villager with a long lifespan, was proven to have strong antioxidant potential through a systematic evaluation in vitro and in vivo. Out of 12 strains, cells, supernatants, and fermented yogurt of *L. mucosae* LMU1001 presented a higher activity of scavenging free radical and total reducing power. *Lactobacillus mucosae* LMU1001 increased antioxidant ability in D-Gal-induced oxidant stressed mice by increasing the activity of GSH-PX and T-SOD, decreasing the level of MDA, and ameliorating the function of the liver through decreasing metabolic enzymes (e.g., ALT, AST, and T-SOD). Furthermore, *L. mucosae* LMU1001 enhanced the expression of *GPX2*, *MT1A*, and *MT1M* in either cell lines of HT-29 and Caco-2, or D-Gal-induced aging mice. The results suggested that *L. mucosae* LMU1001 could be a probiotic candidate, which could be applied in nutraceutical and dairy products to improve the antioxidant status of the host.

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