Effect of Lactobacillus brevis (MG000874) on Antioxidant Related Genes Expression of liver and kidney in d-galactose Induced Oxidative stress Mice Model”

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Abstract

Oxidative stress connected with several stress-associated diseases. Potent radical-scavenging activity of Lactic acid bacteria had been stated due to its ridiculous antioxidant activity. This work designed to find the defensive effects of Lactobacillus brevis MG000874 against oxidative injuries induce by d-galactose in vivo and as well as to explore the gene expression changes in oxidative stress induce mice. D-galactose (D-gal) induction (300mg/BW) in mice raised the levels of SOD (superoxide dismutase) and CAT (catalase) determined by spectrophotometer. In addition, Lactobacillus brevis MG000874 altered gene expression of oxidative stress marker at the messenger RNA (mRNA) levels was determined by RT-PCR. In the mouse model, L. brevis MG000874 significantly improved the GST, GPX, SOD, CAT and B-actin levels in kidney, and liver of the D-gal-induced mice. Moreover, the histological investigation indicated that Lactobacillus brevis MG000874 effectively relieved kidney and liver damage in mice induced by D-gal. L. brevis MG000874 as a powerful antioxidant mediator endorsed fitness and lessened the risk of stress-linked diseases.

Introduction

Aging is a time-dependent irresistible natural physiological decline phenomenon in organism life which insinuates with many physiological and metabolic dysfunctions. Free radical philosophy is one of the most demanding action mechanism in aging cases. Though the manifestation of aging cannot be eluded completely, but it can postpone by scientific approaches (Stroustrup 2018). The aging phenomenon is mysterious and complicated, numerous literature has presented the significant role of oxidative stress in accelerating aging. The stability of reactive oxygen species (ROS) and antioxidants become unstable with the escalation of age, which leads to oxidative damage of lipids, proteins, DNA, and a progressive increase in the chance of mortality and morbidity (Liguori et al. 2018).

It has been proposed that long life may be associated with several elements, for instance inheritances, surroundings and configuration of gut microbiota (Zhao et al. 2011; Biagi et al. 2015). Probiotics comprising Lactobacillus and Bifidobacterium have appealed considerable attention due to their vital role in health promotion. The important purpose of isolating probiotics from all over the world is the production of healthy organisms, particularly with prolonged existence (Woo et al. 2014). The antioxidant capability of lactic acid bacteria (LAB) and their constituents (e.g., proteins and exopolysaccharides) is normally specified and considered as standard for assessment of probiotic functions (Li et al. 2015). Assessment of antioxidant properties comprises in vitro oxidation reaction system (free radicals scavenging, metal ions chelation, and lipid peroxidation inhibition) (Kumar et al. 2019), and in vivo animal confirmation by detecting alteration in the antioxidant enzyme system, e.g. CAT (Catalase), GPX (Glutathione peroxidase), SOD (Superoxide dismutase), and MDA (Malondialdehyde) (Hassani et al. 2018; Lin et al. 2018). Zhang et al. (2010) studied a hyperlipidemic model rat, and demonstrated that Lactobacillus casei helped in reduction of lipid peroxidation by alleviating oxidative stress in liver and serum of the rat. Kanno et al. (2012) reported that Lactobacillus plantarum 7FM10 isolated from plants exhibited superoxide radical and 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging capabilities in vitro. Therefore, selection of LAB having quenching ability of free radicals becomes more significant in investigating the mechanism of antioxidant. Literature revealed that high concentration of D-galactose (D-gal) helped in the generation of ROS by accumulation of advanced glycation end products, as a result oxidative stress and cellular damage produced in the tissues (Shwe et al. 2018; Du et al. 2019). To
date, several reports pertaining to antioxidant evaluation of LAB have been published; however, a systematic evaluation of the antioxidant mechanism from the gene expression level of mice, has been reported rarely. In our previous study, we investigated the L. brevis MG000874's effect on antioxidant enzyme system by inducing d-galactose (Noureen et al. 2019). The current study was therefore to examine the effect of the L. brevis MG000874 on the gene expression of oxidative stress markers in D-gal induced mice.

**Material And Method**

Hydrogen peroxide (H$_2$O$_2$) (Daejung, Siheung-si, Korea), pyrogallol (sigma), D-galactose, (Germany), ascorbic acid (Germany), RNA extraction kit, Syber green master mix, and cDNA extraction kit were procured from local dealer.

**Bacterial culture**

The strains of bacteria were incubated at 37°C by culturing in MRS broth (pH 6.6 ±02) overnight. For further study, bacterial cells were made by following Lin and Yen (1999) method.

**In vivo evaluation of antioxidant prospective**

Sixty days old male albino mice (Mus musculus, Weight: 40± 2 g, n=60) was set aside in cages under schematized circumstances (temperature 22± 2°C, dampness 45± 5%, 12 hour light/ dark cycles). During the experiments, open access was provided for water and food. The mice were indiscriminately distributed after one week of acclimatization into six groups. These groups were negative control group (N): no treatment, probiotic cells, treatment group with L. brevis MG000874 (B), positive control (G): D-galactose treatment group (300mg/BW), ascorbic acid treatment group (A), D-galactose and L. brevis MG000874 treatment group (BG), D-galactose and ascorbic acid treatment group (AG).

**Dose setting**

L. brevis (MG000874) and ascorbic acid were given via gastric gavage (Shen et al. 2011: Noureen et al. 2019). D-gal was injected at the dose of 300 mg kg$^{-1}$ BW/ day through subcutaneous route for 5 weeks; on the behalf of many pilot studies for quick aging process (data not included). Anaesthetized the Animals after five weeks of treatment. Organ index and tissues (Kidney and Liver) were prepared by following Noureen et al (2019).

**Estimation of antioxidants**

The antioxidant level in the liver and kidney were estimated through SOD and CAT. The detailed procedure of above parameter has been described in previous studies (Noureen et al. 2019).

**Histopathological studies**

For the histopathological study, fixed tissues in formalin were processed through Hematoxylin and Eosin staining (Qui et al. 2017). Slides observed in the light microscope having fitted camera (Labomed, USA).

**Extraction of RNA and preparation of CDNA**
RNA was extricated from tissues following TRIzol method (Chomczynski and Sacch 2006). The purity and quantity of attaining RNA (Ribonucleic acid) were estimated through ND-1000 (Nanodrop, Thermo Fisher Scientific). CDNA (Complementary DNA) was made through Synthesis Kit (First-Strand CDNA kit).

**Real-time PCR (Rt PCR)**

RNA expression of SOD, CAT, GST, GPX and β-actin was investigated by means of RT-PCR. Each primer sequence’s detail was provided in Table 1. Concisely, qPCR reaction mixture (20 µl) contained CDNA (01 µl), primer (1.4 µl), SYBR Green master mix (12.5 µl) and nuclease-free water (9.5 µl). 40 cycles of qPCR were done on an RT-PCR system (Bio-Rad CFX): 3 min denaturation at 95 °C, 10 s, annealing at 54-59 °C and 30 s elongation at 72 °C. The expression of mRNA was determined by average quantification cycle (Cq) values. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used as a reference gene (Eissa et al. 2016). The expression was calculated by following

\[
\text{Normalized expression ratio (NER)} = 2^{\Delta\Delta C_t}
\]

\[
\Delta\Delta C_t = \Delta C_t^{(SOD)} - \Delta C_t^{(GAPDH)}
\]

\[
\Delta C_t^{(SOD)} = \text{Sample } C_t^{(SOD)} - \text{reference sample } C_t^{(SOD)}
\]

\[
\Delta C_t^{(GAPDH)} = \text{Control } C_t^{(GAPDH)} - \text{reference of control } C_t^{(GAPDH)}
\]

**Statistical analysis**

One-way ANOVA was performed for group comparison (P-values 0.05, significant: Tukey’s test) by using ‘IBM SPSS 21.0. Tests were done in triplicate and their outcomes were displayed as standard error of means (SEM).

**Results**

**Organ index**

Table 2 revealed that the indexes of liver and kidney in the stress group (G) were significantly lesser than those in the normal group (N: p < 0.05), presenting that induction of d-galactose was the cause of organ stress or aging. However, the liver and the kidney index of treated groups BG and AG were significantly higher than those in the G group showed more observable effects on inhibiting organ aging.

**SOD and CAT activities in Liver and Kidney tissues**

The activities of SOD and CAT in the liver and Kidney of the d-galactose group (G) were the lowest (Figure 1). Following treatment with L. brevis MG000874 (BG), or ascorbic acid (AG), the activities of SOD, and CAT were improved. Particularly, these activities in mice treated with L. brevis MG000874 (B) or ascorbic acid (A) group were very close to normal group (N), illustrating that of L. brevis MG000784 and ascorbic acid were better at delaying the stress factor.

**Histological examination of mice liver and kidney**
The liver photomicrograph was displayed in Figure 2. Liver cell morphology of normal group (N) was consistent, no infection traces found, the size and staining were even, the cell line was organized in sequence and the central vein was spread radially. Although, in comparison to d-galactose group (G), hepatocytes were messy and showed uneven morphology, cell inflammation, deficiency of cell margin and a rough shape of the central vein. Those mice treated with \textit{L. brevis} MG000874 or ascorbic acid displayed less injury. In the \textit{L. brevis} MG000874 treated group (BG), liver damage was less than d-galactose group (G), and thus, the efficacy of \textit{L. brevis} MG000874 in inhibiting liver deterioration was better.

The kidney photomicrograph were shown (Figure 3) that the Bowman's capsule of N and B group had normal distal convoluted tubules, vascular pole, renal parenchyma, inner cellular layer of podocytes and outer squamous capsular cells. Glomerulus had no traces of proteinaceous substances in urinary spaces (Fig 2a & c). In stress group (G), degeneration of renal parenchyma, some cellular necrosis of tubular epithelial and pyknotic nuclei, disappearance of proximal and distal convoluted tubules spaces were detected. Morphology of kidney tissue in \textit{L. brevis} MG000874 treatment group (BG) and ascorbic acid treatment group (AG) was significantly improved and revealed similar micrographs as normal group (N).

\textbf{Liver gene expression content}

SOD, CAT, GST, GPX and B-actin gene expression of liver tissues was determined by RT-PCR (Figure 4). In normal control group (N), the expression levels of SOD, CAT, GST, GPX and B-actin were the highest. However, in the d-gal group (G), the mRNA expression levels of the above genes were reversed. In treatment group BG and AG, the expression of SOD, CAT, GST, GPX and B-actin was significantly improved (p < .05).

\textbf{Kidney gene expression content}

The gene expression level of Kidney tissue was presented in Figure 5. The mRNA expression of SOD, CAT, GST, GPX and B-actin of the normal group (N) in comparison to d-gal group (G) was lowest. In treatment group with ascorbic acid (AG), or \textit{L. brevis} MG000874 (BG), the mRNA expression of SOD, CAT, GPX, GST and B-actin was increased. The expression of \textit{L. brevis} MG000874 alone treatment group (B) was highest in comparison to N group, which was stronger than that of ascorbic acid.

\textbf{Discussion}

Lactic acid bacteria (LABs) are very important acidic bacteria, renowned their distinct physiological roles, have special enzymes, and produces many substances during fermentation. They have a great influence on human life and are commonly utilized in many industries like poultry, livestock and food production (Landete et al. 2017; Ramos et al. 2020). LABs strains isolated from different sources like plants and animals that may have antioxidant activity (Kenfack et al. 2018; Rezaei et al. 2020; Unban et al. 2021). This trait is strain and species specific. Therefore, we aimed at finding the antioxidant potential of the \textit{L. brevis} MG000874 strain isolated from animal. In the mice model, the constant sub cut d-galactose injection has been revealed to stimulate stress by increase production of the free radicals and a depletion of the antioxidant activity leading to oxidative stress. The stress-affiliated mechanisms may be damaged by the extraordinary production of reactive free species (Ge et al. 2021). Thus, we designated the d-galactose-induced oxidative stress mice...
model to find the effects of the L. brevis MG000874 as antioxidant on gene expression of stress markers in liver and kidney. In our previous study, we injected the 150mg/Kg/body weight d-galactose in mice for inducing oxidative stress at eight weeks (Noureen et al. 2019). In the current study, we selected the high dose of d-galactose 300mg/kg/BW for quicker result. At this dose the aging sign and symptoms was appeared within four weeks in mice model. An important and basic indicators in biomedical research for observing the stress effects are the organ index in mice. In the stress body, atrophy of the liver is more noticeable (Xu et al. 2016). An immune organ of animal is liver and its atrophy may source of immune deficiency (Knolle and Gerken 2000). Literature revealed that the liver index of oxidative stress mice was considerably decreased as compared to the normal mouse liver (Sang et al. 2017). The metabolic organ is the kidney, and a decrease in kidney index has a great influence on the metabolic ability (Xu et al. 2018). Thus, alterations in the organization of mouse organ can be noted in organ index and are significantly important for evaluating the successful induction of oxidative stress in mice (Bonthius et al. 2015; Sang et al. 2017). The outcomes of this study presented that d-gal caused the atrophy of organs and induction of oxidative stress in mice. Oxidative stress processing was efficiently postponed by treating with L. brevis MG000874 (10^9 cfu/kg), proposing the noticeable anti-stress effect of L. brevis MG000874. Histopathological inspection is the microscopic study of the cells, tissues or organ morphology in order to verify the body changes (Di et al. 2016; Qian et al. 2018). This study revealed that liver and kidney tissues of the d-gal group in comparison to normal group were deteriorated. In treatment with L. brevis MG000874 (BG) or ascorbic acid (AG), the morphological abnormalities of the liver and kidney tissues was considerably upgraded, and the upgrading impact of L. brevis MG000874 was the most noticeable. Tissue morphology of L. brevis MG000874 treated group was very close to the normal group (N).

During the process of bio-oxidation, a huge quantity of ROS is generated in the body. The stability of ROS is attained by numerous antioxidant defence systems, containing, SOD, CAT, GST, GSH, and vitamins. Their synergistic influence transforms excessive ROS into O_2 and H_2O_2 molecules (Zhang et al. 2008). LABs have specific antioxidant ability for quenching free radicals, which can support the antioxidant enzymes. Antioxidant activity is a strain specific feature. Additionally, during the metabolic process of the body, LABs prevent the oxidation process by discharged of antioxidant enzymes such as SOD (Lobo et al. 2010; Feng et al. 2016). In this study, the L. brevis MG000874 significantly elevated the SOD activity and CAT activity in kidney and liver tissue of the treated mice. The experimental outcomes confirmed that the induction of d-gal causes the cellular oxidative stress, resulted in decrease SOD and CAT activity in liver and kidney tissues. Ascorbic acid or L. brevis MG000874 was good in the antioxidant effect during the measurement of SOD and CAT stress indicators in tissues.

To further enlighten the antioxidant, this research has observed the changes in SOD, CAT, GST, GPX and B-actin expression levels of. In animals, SOD is universally expressed as mitochondrial antioxidant enzyme and helps in controlling free radical’s production by keeping healthy (Zhao et al. 2018). CAT functions as scavenging O^- free radicals and stimulating the disintegration of H_2O_2 by preventing oxidation damage (Selvaratnam and Robaire 2016). Literature revealed that the oxidative stress causes the tissue atrophy by dropping SOD and CAT expression (Hart et al. 2015). The outcomes of this study revealed that mRNA expression levels of SOD and CAT in the liver and kidney tissues of the d-gal control group were significantly reduced (p < 0.05), representing that d-gal subcutaneous injection in mice might cause oxidative stress. Though, mRNA
expression levels were significantly improved in treatment with L. brevis MG000874 (p < 0.05). GST is a broad spectrum antioxidant. GPx may be used to regulate the intracellular hydro peroxide level in the gastrointestinal tract (Yan and Chen 2006). β-actin is main structural proteins of cytoskeleton which play a significant part in retaining the shape of the cell (Mousa et al. 1979). We found that GST, GPX and β-actin expression levels were positively upregulated by L. brevis MG000874 in d-gal-induced model mice. These reports indicated that L. brevis MG000874 could perform as antioxidant which might directly hunt ROS.

Lastly, histological investigation verified that cellular inflammation, and apoptosis of hepatocytes were greater in the d-gal alone group and treatment with L. brevis MG000874 showed normal hepatocytes with congested sinusoids in liver. While in kidney, disintegration of renal parenchyma, some cellular necrosis and vanishing of tubules spaces were identified in the stress group as compared to L. brevis MG000874 treatment group (BG). L. brevis MG000874 (probiotic) possessed a renal hepatic protective influence through raising activities of antioxidant enzymes (i.e., SOD, CAT) on d-gal inducted stress (BG) and maintaining the cellular structure. These consequences were also connected to the stimulation of the antioxidant defence system by boosting SOD, CAT, GST, GPX and β-actin controlled gene expression. Our findings further proposed that L. brevis MG000874 could maintain the intracellular redox balance in the livers and kidney of D-gal-treated mice by renewing the activities of antioxidant enzymes.

Conclusion

The current investigation found that L. brevis MG000874 has an antioxidants effect. Induction of D-gal initiated the oxidative stress, reduced the antioxidant enzyme activity. Supplementation of L. brevis MG000874 or ascorbic acid resulted in body weight gain, and organ index, restored the antioxidant defense system by increasing the antioxidant enzyme activity and upregulating the gene expression. As a whole, these finding verified that L. brevis MG000874 promoted the antioxidative gene expression and cellular antioxidative responses in vivo. Therefore, L. brevis MG000874 will have the potential to be further explored as an antioxidant functional food in the prevention of more stress-related diseases.

Declarations

Authors Contribution

Saleha Noureen: conceptualization, methodology, analysis, data curation and writing - original draft. Tanveer Hussain: Analysis, Aasma Noureen: reviewing and editing, Ahmed E. Altyar: reviewing and editing.

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Data availability

All data generated or analyzed during this study are included in this published article (and its Supplementary information files).
Ethics approval and consent to participate: The protocol for these experiments was approved by the Animal Ethics Committee of Virtual University of Pakistan.

Consent for publication: Not applicable

Competing interests: The authors declare no competing interests

References


## Tables

### Table 1

**Target genes and reference gene’s primer set**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sets</th>
<th>Annealing Temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total SOD</td>
<td>(F) 5 AGGATTAACTGAAGGCAGCAT 3</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>(R) 5 TCTACAGTTAGCCAGGCAGCAG 3</td>
<td></td>
</tr>
<tr>
<td>CAT</td>
<td>(F) 5 ACGAGATGACACACTGTGACAG 3</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>(R) 5 TGGGTGCTCTTGTCGATG 3</td>
<td></td>
</tr>
<tr>
<td>GPX</td>
<td>(F) 5 AAGGTGCTGCTGTCAGGGAATG 3</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>(R) 5 CGTCTGGACCTACCAGAATCT3</td>
<td></td>
</tr>
<tr>
<td>GPX</td>
<td>(F) 5 AAGGTGCTGCTGTCAGGGAATG 3</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>(R) 5 GTCCTGGACACGTCAACTT3</td>
<td></td>
</tr>
<tr>
<td>ß-ACTIN</td>
<td>(F) 5 CTCTCAGCTGTGATGGAATG 3</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>(R) 5 AGCCATGTAGCTAGCCTCC 3</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>(F) 5 TGCAGTGGCAAAGTGGAGAT 3</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>(R) 5 TTTGCCCCGTGGAGGTGATC 3</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2

**The effect of treatment of organ index**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Parameters</th>
<th>N</th>
<th>B</th>
<th>A</th>
<th>G</th>
<th>BG</th>
<th>AG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Body Weight</td>
<td>40.00 ± 1.15(^a)</td>
<td>41.66 ± 2.23(^a)</td>
<td>41.02 ± 0.88(^a)</td>
<td>33.00 ± 0.57(^b)</td>
<td>37.66 ± 0.66(^ab)</td>
<td>37.01 ± 0.88(^ab)</td>
</tr>
<tr>
<td></td>
<td>Kidney index</td>
<td>0.24 ± 0.01(^ab)</td>
<td>0.28 ± 0.01(^a)</td>
<td>0.27 ± 0.01(^ab)</td>
<td>0.20 ± 0.01(^c)</td>
<td>0.24 ± 0.01(^ab)</td>
<td>0.23 ± 0.01(^bc)</td>
</tr>
<tr>
<td></td>
<td>Liver index</td>
<td>5.95 ± 0.76(^b)</td>
<td>6.33 ± 0.08(^a)</td>
<td>6.23 ± 0.08(^a)</td>
<td>4.9 ± 0.06(^c)</td>
<td>5.55 ± 0.12(^b)</td>
<td>5.53 ± 0.12(^b)</td>
</tr>
</tbody>
</table>

Data represent Mean ± SEM. Values having different superscript alphabet letter in same tier significantly vary at \( P < 0.05 \). N: normal group, G: d-galactose treatment group, A: ascorbic acid treatment group, B: *L. brevis* MG000874 treatment group, BG: galactose and *L. brevis* MG000874 treatment group, AG: galactose and ascorbic acid treatment group.
Figures

Figure 1

Effects of L. brevis MG000874 and ascorbic acid on D-Gal induced changes in SOD and CAT of liver and kidney. B: L. brevis MG000874 group. G: d-gal control group, A: Ascorbic Acid group, BG: group getting both D-Gal and L. brevis MG000874, AG: Group getting Ascorbic acid and D-Gal. Data represent Mean ± SEM. Values having different superscript letter (a, b, c, d) significantly vary at $P < 0.05$. 
Figure 2


Figure 3

Figure 4

Effects of L. brevis MG000874 on D-Gal induced changes of the expression level of SOD, CAT, GST, GPX and B-actin in liver. B: L. brevis MG000874 group. G: d-gal group, A: Ascorbic Acid group, BG: group getting both D-Gal and L. brevis MG000874, AG: Group getting Ascorbic acid and D-Gal. Data represent Mean ± SEM. Values having different superscript letter (a, b, c, d) significantly vary at $P < 0.05$. 
Figure 5

Effects of *L. brevis* MG000874 on D-Gal induced changes on the expression of SOD, CAT, GST, GPX and B-actin in kidney. B: *L. brevis* MG000874 group. G: Positive control group, A: Ascorbic Acid group, BG: group getting both D-Gal and *L. brevis* MG000874, AG: Group getting Ascorbic acid and D-Gal. Data represent Mean ± SEM. Values having different superscript letter (a, b, c, d) significantly vary at $P < 0.05$. 