Origina Article

Pre- and Post-Treatment Effects of *Lactobacillus acidophilus ATCC* 4356 on Oxidative Stress and Function of Liver in Diabetic Male Rats

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ABSTRACT

Background:
This study investigated the effects of *lactobacillus acidophilus ATCC4356* on the oxidant and antioxidant factors of the liver and levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) before and after streptozotocin-induced diabetes in male rats.

Materials and Methods:
Thirty male Wistar rats were divided into five groups (n=6): Control (C), Control probiotic (CP), Diabetic (D), Diabetic Pretreatment with lactobacillus (DPB), and Post-treatment with lactobacillus (DPA) groups. C group received daily 1 mL of normal saline for 6 weeks. CP group received daily 1×10^9 cfu/mL *L. acidophilus ATCC 4356* for 6 weeks. D group received daily 1mL normal saline for 2 weeks before and for 4 weeks after diabetes induction. DPB group received daily 1×10^9 cfu/ml *L. acidophilus ATCC 4356* for 2 weeks before and for 4 weeks after diabetes induction. DPA group first received daily 1mL normal saline for 2 weeks before diabetes and then received daily 1×10^9 cfu/mL *L. acidophilus ATCC 4356* for 4 weeks after it.

Results:
L. acidophilus ATCC 4356 decreased liver malondialdehyde (MDA) and H_2O_2 concentration and serum AST significantly in both pre- and post-treatment groups compared with the D group. Catalase activity (CAT) and serum ALT showed a significant decrease in the post-treatment group compared with the D group. Glutathione peroxidase (GPx) activity showed a significant increase in the post-treatment group compared to the D group.

Conclusion:
The present study showed that *L. acidophilus ATCC4356* had more protective effects on the liver in the post-treatment group compared with the pretreatment one.

Keywords: Diabetes, *Lactobacillus acidophilus,* Hepatic complications, Oxidative stress, ALT, AST.

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INTRODUCTION

More than 300 million people in the world suffer from diabetes mellitus (Diabetes), and their number is increasing. It is expected to rise in the future (1, 2). Diabetes usually has a variety of complications, such as retinopathy, neuropathy, nephropathy, and cardiovascular disease (3).

Increased blood glucose in this disease, by activating cascade reactions, is a cause for the production of free radicals and consequent oxidative stress in various tissues of the body (4). Free radicals, due to the ability to induce chemical reactions with the oxidation of lipids, nucleic acids, proteins, and carbohydrates, accelerate the onset of clinical complications and result in tissue damage in patients (5).

The liver is a complex and large organ whose main role is to design and manage the metabolism of carbohydrates, proteins, and lipids (6). Liver and kidney failure are the most common causes of death in patients with diabetes (7). The liver enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyltransferase (GGT), are routinely used in the evaluation of liver function. AST and ALT are considered markers of hepatocellular health, whereas GGT also indicates biliary tract function (8). Along with damage and impaired liver function, liver enzymes (ALT, AST, and GGT) are released into the bloodstream due to damage to the cell membrane (9). In diabetes, there is a decrease in liver weight due to enhanced catabolic processes such as glycogenolysis, lipolysis, and proteolysis (10). In a study that investigated hepatic morphological changes and oxidative stress in chronic streptozotocin-induced diabetic rats, increased serum levels of ALT and AST and tissue levels of superoxide dismutase activity and decreased catalase (CAT) and glutathione peroxidase (GPx) activity was observed compared to the control group (11). Antioxidants act as important defenses against toxicity-producing oxidants. Therefore, considering the role of free radicals in diabetes, one of the areas of research in controlling this disease is the reduction of oxidative agents (12). The formation of a number of antioxidant drugs for the prevention and treatment of diabetes has evolved over the past three decades (13).

Probiotics are among the microorganisms used to treat diabetes with some antioxidative properties. Probiotics are non-pathogenic microorganisms that, if they are used in adequate amounts, have beneficial effects on their host by creating a microbial balance in the intestine (14). Lactobacilli and bifidobacteria are important probiotic strains useful in the promotion of human health (15). Several beneficial properties of probiotics are as follows: helping to cure lactose intolerance, diarrhea, constipation, allergies, inflammatory bowel disease, irritable bowel syndrome, gastric ulcer, immune system stimulation, autoimmune disease prevention, a decrease of cholesterol, and anti-cancer property (16-18). Amdekar et al., in a study conducted in 2012 on the protective effect of lactobacillus on bone damage and antioxidant status of liver and kidney in male Wistar rats, concluded that the use of Lactobacillus acidophilus significantly increased the level of superoxide dismutase enzyme, and consumption of Lactobacillus acidophilus and Lactobacillus casei significantly increased the level of glutathione peroxidase (GPx) (19). In another study, treatment with probiotics and vitamin C in alloxan-induced diabetic Wistar rats was investigated, and ameliorated oxidative stress parameters were observed (20). Previously, we showed the effects of Lactobacillus acidophilus ATCC4356 on the reproductive system in diabetic male rats (21).

Considering the high prevalence and irreparable complications of diabetes and the role of free radicals in the development and progression of this disease, our objective is to investigate the role of probiotic *Lactobacillus acidophilus ATCC4356* in the control of diabetes by measuring oxidative stress parameters such as hydrogen peroxide (H_2O_2), malondialdehyde (MDA), antioxidant factors such as catalase (CAT) and GPx activities in the liver tissue as well as serum levels of serum ALT and AST in normal and diabetic rats both before and after diabetes induction.

MATERIALS AND METHODS

Preparation of bacteria

Lactobacillus acidophilus ATCC 4356 was purchased as a lipophilic powder from Zist Kavosh Iranian Co cultured in MRS Medium (De Man, Rogosa and Sharpe agar), and incubated at 37°C for 24 hours under anaerobic conditions. Following that, it was centrifuged for 10 minutes at 6000

rpm. After this procedure, the medium was poured onto the bacteria and concentrated on normal saline. Also, the number of Lactobacillus acidophilus (ATCC4356) colonies in the experimental group was counted.

Animals

30 male Wistar rats (230-240 g weight) were purchased from Kerman University of Medical Sciences. Animals were maintained one week before the experiment to adapt to the new environment in the animal house of the Faculty of Science of the Department of Biology of the Shahid Bahonar University of Kerman with 12 hours of darkness and 12 hours of lighting at 23° C and did not have any restrictions for food and water.

Experimental Design:

Streptozotocin (STZ, sigma, 60mg/kg body weight) was used to induce diabetes. Freshly prepared STZ (dissolved in cold normal saline) was administered intraperitoneally. 72 hours after STZ injection, fasting serum glucose levels were measured using a Medisense Optium glucometer, and rats with blood glucose levels above 300 mg/dL were considered diabetic.

Rats were divided into five groups as follows (n=6):

1- Control group (C): In addition to the usual diet, they received 1 mL of normal saline as gavage daily for 6 weeks.
2- Control probiotic group (CP): In addition to the usual diet, they received 1 mL of Lactobacillus acidophilus ATCC4356 daily for 6 weeks.
3- Diabetic group (D): They were initially gavaged with normal saline for 2 weeks, and after induction of diabetes, they received normal saline as gavage for 4 weeks.
4- Probiotic pretreatment diabetic group (DPB): At first, they received *L.acidophilus ATCC4356* for 2 weeks, and after induction of diabetes, they were gavaged with *L.acidophilus ATCC4356* for 4 weeks.
5- Probiotic post-treatment diabetic group (DPA): They were initially gavaged with normal saline for 2 weeks, and after induction of diabetes, they were gavaged with *L.acidophilus ATCC4356* for 4 weeks.

At the end of the experiment, first deeply anesthetized with CO_2, and then assassinated by giyotin. Livers were removed immediately and prepared for oxidant and antioxidant assays. Blood serum was separated for ALT and AST assay.

ALT and AST assay

To measure liver enzymes in serum, we used the kit of ziestchem company. In the manual reagent kit, there is the relevant amino acid alanine or aspartate along with pyridoxal phosphate (PLP). By adding the reagent to the sample, aminotransferases present in the sample convert the amino acid in the presence of PLP to the corresponding α-ketoacid pyruvate or oxaloacetate; therefore, the amount of pyruvate or oxaloacetate produced is equal to the amount of ALT and AST.

Investigation of oxidative stress of liver

Protein assay

Bradford method was used (1976) for protein measurement. In this method, 0.5 g of liver tissue was homogenized in 50 mM phosphate buffer and then centrifuged at 10000 g for 10 min at 4°C. The resulting tissue extract was added to 5 ml of biuret solution. After 25 min, absorbance was read at 595 nm (22).

CAT assay

CAT activity was measured by the method of Aebi (23). To a cuvette containing 1.5 ml of the reaction mixture (H_2O_2+50 mM phosphate buffer), the 100µl tissue extract was added. The reaction was started by the decomposition of H_2O_2, and CAT activity was measured spectrophotometrically at 240 nm.

GPx assay

GPx activity was measured by the method of Plewa *et al.* (24). To a cuvette containing 2.5 ml of the reaction mixture (H_2O_2+50 mM phosphate buffer+guaiacol), 20µl of tissue extract was added. The reaction was started by the oxidation of guaiacol, and GP_X activity was measured at 470 nm.

MDA assay

Thiobarbituric Acid Reactive Substances (TBARS) level, measured as an index of malondialdehyde (MDA) production and hence lipid peroxidation, was assessed

in the tissues by the method of Heath and Packer (25). In brief, tissue extract (1 mL) was added to test tubes containing 4 ml of TCA 20% containing TBA 0.5%, and the reaction mixture was heated at 95°C for 30 min and, after cooling, centrifuged at 10000 g for 10 min and MDA-TBA complex was measured at 532 nm.

H_2O_2 assay

H_2O_2 level, measured as an index of oxidant factors, was assessed in the tissues by the method of Velikova *et al.* (26). Tissue was homogenized in 1 mL TCA. The homogenate was centrifuged at 10000 g for 10 min at 4°C. H_2O_2 concentration was measured in a cuvette containing 0.5 ml of tissue extract and 0.5 ml phosphate buffer 10 mM (pH=7.4), and 1 ml of potassium iodid 1 mM was added, and H_2O_2 concentration was measured at 390 nm.

Statistical analysis

Data were expressed as mean±SEM. Statistical differences between the groups were analyzed using the one-way analysis of variance (ANOVA) test and TUKEY post-test with SPSS software version 16. $P<0.05$ was considered significant.

RESULTS

Table 1 shows the effects of *L. acidophilus ATCC4356* at 10^9 cfu/mL/day dose on fasting blood glucose, body, and liver weight of STZ-induced diabetic rats after 42 days.

Fasting blood glucose concentration in D, DPB, and DPA groups was significantly higher compared with C and CP groups ($P<0.001$). Also, DPB and DPA groups showed a significant increase in glucose concentration compared with D group ($P<0.001$). The body weight of the D, DPB, and DLA groups was significantly lower compared with C and CP groups ($P<0.001$). Furthermore, DPB group showed a significant decrease in body weight compared with D group ($P<0.05$). The liver weight in the D and DPB groups was significantly lower compared with C and CP groups ($P<0.01$). DPA group showed a significant decrease in liver weight compared with C and CP groups ($P<0.05$ and $P<0.001$, respectively).

Table 2 shows the effects of *L. acidophilus ATCC4356* at 10^9 cfu/mL/day dose on MDA and H_2O_2 levels and the activity of GPx, CAT, ALT, and AST enzymes of STZ-induced diabetic rats after 42 days.

MDA concentration in the D group is significantly higher compared with the others. H_2O_2 concentration in the D group is significantly higher compared with the C, and CP groups ($P<0.001$). Also, H_2O_2 concentration in DPB and DPA groups showed a significant decrease compared with the D group ($P<0.01$ & $P<0.05$, respectively).

GP_X activity in D, CP, DPB, and DPA groups is significantly lower compared with the C group ($p<0.001$). Also, the DPA group shows a significant increase compared with the diabetic group.

CAT activity in the D group is significantly higher

Table 1. The effect of administration of probiotics on fasting blood glucose, body weight, and liver weight in experimental groups.

Variables	Group C	Group CP	Group D	Group DPB	Group DPA
Glucose concentration (mg/dL)	49 ± 1.9	65.50 ± 5.2	324.86 ± 23.5 *** ‡‡‡	544.14 ± 25.8 *** ### ‡‡‡	576.86 ± 14.8 *** ### ‡‡‡
Body weight (gr)	262.33 ± 2.8	273.33 ± 2.0	178.43 ± 5.9 *** ‡‡‡	160.43 ± 3.8 *** # ‡‡‡	171.43 ± 5.07 *** ‡‡‡
Liver weight (gr)	7.8667 ± 0.20	8.3000 ± 0.27	5.9000 ± 0.32 *** ‡‡‡	5.6657 ± 0.12 *** ‡‡‡	6.6625 ± 0.25 * ‡‡‡

Values are mean±SD for six rats. Values with different superscripts differ significantly ($P<0.001$), ($P<0.01$), and ($P<0.05$).
***and * Significant difference with C group ($P<0.001$ and $P<0.05$, respectively).
and # Significant difference with D group ($P<0.001$ and $P<0.5$, respectively).
‡‡‡Significant difference with CP group ($P<0.001$).

Table 2. Probiotic effects on MDA and H_2O_2 levels and activity of GPx, CAT, ALT, and AST enzymes in experimental groups

Variables	MDA concentration (mol/gr fw)	H_2O_2 concentration (mol/gr fw)	GPx activity (U/mg protein)	CAT activity (U/mg protein)	ALT concentration (U/L)	AST concentration (U/L)
C	0.053±0.005	0.10±0.018	2.08±0.63	0.13±0.04	91.6±12.27	196.09±9.24
CP	0.15±0.046	0.16±0.028	0.76±0.039 $$$	0.13±0.034	64.12±5.45	188.29±8.75
D	0.35±0.031 ***	0.34±0.034 ###	0.23±0.01 $$$	0.42±0.098 #	130.8±3.57 ##	260±9.18 # $
DPB	0.06±0.005	0.17±0.025 **	0.42±0.14 $$$	0.27±0.061	107.23±10.27 ¥ $$	201.46±16.28
DPA	0.10±0.016	0.21±0.024 *	1.22±0.31 $$$ *	0.14±0.049	73.43±4.21	192.11±14.93

C: Control group, CP: Control probiotic group, D: Diabetic group, DPB: Probiotic pretreatment diabetic group, DPA: Probiotic post-treatment diabetic group
Values with different superscripts differ significantly ($P<0.001$), ($P<0.01$), and ($P<0.05$)
***Significant difference with C, CP, DPB, and DPA groups ($P<0.001$)
** Significant difference with D group ($P<0.01$)
* Significant difference with D group ($P<0.05$)
Significant difference with C and CP groups ($P<0.001$)
Significant difference with C group ($P<0.01$)
Significant difference with C, CP, and DPA groups ($P<0.05$)
¥¥¥ Significant difference with CP and DPA groups ($P<0.001$)
¥ Significant difference with the DPA group ($P<0.05$)

compared with C, CP, and DPA groups ($P<0.05$).

Serum ALT level in the D group is significantly higher compared with C and CP groups ($P<0.01$& $P<0.001$, respectively). Also, the DPB group is significantly higher compared with CP and DPA groups ($P<0.01$& $P<0.05$, respectively). The DPA group shows a significant decrease compared to the D group ($p<0.001$).

Serum AST level in the D group is significantly higher compared with C, CP, and DPA groups ($P<0.01$). DPB group showed a significant decrease compared with D group ($P<0.05$).

DISCUSSION

In this study, we observed that in the diabetic group, the antioxidant enzyme activity of CAT and GPx increased and decreased, respectively. Also, in this group, the level of H_2O_2, MDA, ALT, and AST increased compared with the control group. Treatment with probiotics had ameliorative effects, but on the whole, there were no significant changes between pre- and post-treatment groups.

Insulin-dependent diabetes mellitus is characterized by a series of complications that affect many organs. During diabetes, persistent hyperglycemia causes increased production of OFRs through autoxidation of glucose (27) and also by non-enzymatic protein glycation (28). Oxygen free radicals (OFRs) have been implicated in the pathogenesis of diabetes mellitus (29). Antioxidant enzymes (CAT, GPx, SOD) offer protection to cells and tissues against oxidative injury (30).

In this study, diabetic animals showed an increase in levels of MDA, H_2O_2, CAT activity, and serum ALT and AST concentration, while GP_X activity decreased. In agreement with this, Yeul Cho et al. showed that in the liver of diabetic rats, MDA level and CAT activity increased while GPx activity decreased (31). Also, in coordinating with this result, Kamalakkannan et al. observed that in the liver of diabetic rats, MDA level increased and GPx

activity decreased; however, catalase activity decreased (32). In another study, Ramazan Yilmaz et al. observed that in the liver of diabetic rats, MDA level and CAT and GPx activity increased (33). Ostovan et al., in their study on the antioxidant activity of the *Citrullus colosynthis* pulp on oxidative stress factors of liver tissue, observed that in diabetic rats, MDA and H_2O_2 levels and CAT activity increased while GPx activity decreased (34).

Several studies with human and animal models using a thiobarbituric acid reactive substances (TBARS) assay (35-37) have shown increased lipid peroxidation in membranes and lipoproteins in diabetic states. The increased concentration of TBARS suggests an increase in oxygen free radicals (OFRs) that could be due to either their increased production or decreased removal (38). CAT has a role in the detoxification of H_2O_2 and the breakdown of H_2O_2 to H_2O (39). Therefore, the increase in CAT might be due to an increase in H_2O_2. Low GPx activity in diabetic tissues might be due to low GSH content because GSH is a substrate and cofactor of this enzyme (40). In the process of catalyzing H_2O_2 to H_2O, GPx converts GSH to GSSG, which by GRx is reduced to GSH (41).

Recently the role of the gut microbiota as a modulator of metabolic and inflammatory processes has been investigated. Intake of probiotics is a safe alternative for normalizing the gut microbiota (42). In an in-vitro study, the antioxidative potential of intestinal lactic acid bacteria *L. acidophilus* ATCC 4356 was reported (43).

In the current study fasting blood glucose in pre- and post–treatment groups with *Lactobacillus acidophilus ATCC4356* had a significant increase in the diabetic group. These results were in agreement with the study of Yadav et al. that evaluated the effects of oral administration of probiotic dahi containing *Lactobacillus acidophilus* and *Lactobacillus casei* for 15 weeks on gastropathy consequences in streptozotocin-induced diabetic rats and it did not change the blood glucose levels in chronic hyperglycemic conditions but reduced the oxidative stress markers such as TBARS in intestinal tissues and glycosylation of hemoglobin (44). On the other hand, there are several studies in which probiotics decreased blood glucose levels (45-47). These differences in the functions of several lactic acid bacteria might be caused by structural differences between species or strains of bacteria (48).

In this study, post-treatment group with *L. acidophilus ATCC 4356* showed a significant decrease in CAT activity and serum ALT level and a significant increase in GPx activity compared with the diabetic group, while levels of MDA and H_2O_2 and serum AST levels in both pre- and post-treatment groups showed a significant decrease compared with diabetic group. Coordinate with these results, Kumar et al. showed that using *Lactobacillus fermentum* strain RS-2 on alloxan-induced diabetic rats increased CAT, SOD, and GPx activity in the liver (49). In another study, Sharma et al. showed that administration of Lactobacillus casei and Bifidobacterium bifidum returned the antioxidant indices in the pancreas of diabetic rats to a normal level with a reduction in lipid peroxidation and elevation in reduced GSH, SOD, CAT, GPx, GR, and glutathione-S-transferase (47). In another study that investigated the protective role of supplements with foreign *Bifidobacterium* and *Lactobacillus* in experimental hepatic ischemia-reperfusion injury, decreased serum ALT and hepatic MDA and increased SOD activity in the liver were observed, and it also markedly ameliorated liver histopathology (50).

Ejtahed et al., in another study, showed that probiotic yogurt containing *Lactobacillus acidophilus La5* and *Bifidobacterium lactis Bb12* for 6 weeks significantly decreased hemoglobin A1c and increased GPx activities and total antioxidant status in type 2 diabetic patients (51).

The mechanisms of antioxidant activity of probiotics have not been properly understood. A study of molecular mechanisms and in-vitro antioxidant effects of Lactobacillus plantarum MA2 showed that L.plantarum MA2 could tolerate hydrogen peroxide up to 2 mM, and its fermentate (fermented supernatant, intact cell, and cell-free extract) had strong reducing capacities, lipid peroxidation inhibition capacities, Fe2+-chelating abilities, as well as various free radical scavenging capacities. Additionally, both the fermented supernatant and cell homogenate exhibited GPx activity and SOD activity (52).

Also, another study examined some of the antioxidant properties of probiotics for the following reasons:

1. Probiotics chelate metal ions. 2. Probiotics possess their own antioxidants. 3. Probiotics produce antioxidant

metabolites. 4. Probiotics up-regulate the antioxidase activities of the host. 5. Probiotics increase levels of antioxidant metabolites of the host. 6. Probiotics regulate signaling pathways. 7. Probiotics down-regulate the activities of enzymes producing ROS. 8. Probiotics regulate intestinal microbiota (53).

CONCLUSION

Considering the results of this study, administration of *Lactobacillus acidophilus ATCC4356* in the post-treatment group had more effects in amelioration of antioxidant status compared with the pretreatment group, and its mechanism probably is different from known mechanisms.

COMPETING INTERESTS

The authors declare no conflict of interest related to this work.

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