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Research Article

IN VITRO ANTIOXIDANT AND *IN VIVO* ANTIDIABETIC ACTIVITY OF TWO POTENTIAL PROBIOTIC *ENTEROCOCCUS* SPP. ON ALLOXAN-INDUCED DIABETIC RATS

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ABSTRACT

Objective: *In vitro* antioxidant activity, *in vivo* antidiabetic property and intestinal attachment by two potential probiotic bacterial strains, namely, *Enterococcus faecium* and *Enterococcus hirae* were studied using albino rats.

Methods: Antioxidant the activity was assessed using 2,2-Diphenyl-1-picrylhydrazyl radicals scavenging assay. Alloxan was administered intraperitoneally to induce diabetic conditions in experimental rats. Animals were treated with oral administration of *Enterococcus* spp., such as *E. faecium,* and *E. hirae* isolated from goat and sheep milk. The control animal group received normal saline for the same days. Glibenclamide drug was used as a positive control against probiotic bacterial cells.

Results: However, administration of probiotic bacterial strains *E. faecium* and *E. hirae*, in albino rats significantly ($p<0.05$) at varying doses lowered blood glucose levels in diabetic rats as compared to the diabetic control group. Both the species of *Enterococcus* increased the bodyweight of experimental rats. However, *E. faecium* was the best antidiabetic strain having the antioxidant activities also in comparison to *E. hirae.* The attachment of probiotic bacterial cells *E. faecium* on the rat's intestine wall against pathogens was examined. Furthermore, *E. faecium* showed its aggregation with pathogens by attachment of the intestines of albino rats. This showed that both the bacterial strains exhibited *in vivo* antidiabetic effect.

Conclusion: The results of this study showed that probiotic bacteria possess antioxidant, antidiabetic activities, and attachment of intestine.

Keywords: Probiotics bacteria, Lactic acid bacteria, *Enterococcus* spp., Antidiabetic effect, Antioxidant activity, Intestinal attachment

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INTRODUCTION

Diabetes is one of the common metabolic disorders throughout the world. It is a major health problem affecting millions of people of all gender and age groups worldwide. Diabetes occurs when the body is unable to produce enough insulin or has complications of its proper use [1]. Therefore, it is a multifactor disorder. Defects in reactive oxygen species (ROS) scavenging enzymes, deficiencies or disruptions in insulin secretion, and high oxidative stress damaging pancreatic beta cells will result in chronic hyperglycemia and troublesome disruptions in carbohydrate, fat, and protein metabolisms [2]. The beta-pancreas cells are responsible for insulin production in the human body. Insulin can attach to the cell surface by specific ligands and glucose absorption, increase by cell and blood glucose decrease. Therefore, beta pancreas dysfunctions reducing the insulin level in the blood can cause hyperglycemia disease and blood accumulation [3]. Various studies have demonstrated that hyperglycemia-induced generation of free radicals mediates mainly oxidative stress and contributes to the development and evolution of diabetes [4]. Pancreatic β-cells are mainly susceptible to the harmful effects of ROS because of its low expression of the genes of antioxidants as compared to other tissues. Thus, the damage of β-cells is caused by an increase of ROS through the apoptosis-induction and insulin-biosynthesis suppression [5].

The probiotics are "live Microorganisms conferred a health benefit on the host when they are administrated in adequate amounts" [6]. Thus, the preventive effects of probiotic lactic acid bacteria (LAB) on diabetes have recently been demonstrated in several experimental studies. Various antidiabetic agents are available in the market, but their uses have become limited due to severe adversary effects in humans, such as diarrhea, flatulence, and bloating. Hence, there is much interest in using probiotics as health supplements [7]. One of the most important criteria for the selection of these types of strains is the ability of probiotic bacteria to adhere to intestinal surfaces. Attachment of probiotic bacteria to mucosa also influences the host's normal microbiota and gastrointestinal immune system [8]. The most important factors are the attached of probiotics to intestinal epithelial cells and colonization of the intestinal tract because of their importance for maintaining the balance in the normal gut microbiota of the host and affect the ability of the bacterial species to function as a probiotic [9]. *Enterococcus faecium* and *Enterococcus hirae* were previously isolated from goat and sheep milk and evaluated for its probiotic properties, including acid and bile tolerance and production of antimicrobial compounds [10]. The present study was undertaken to investigate the *in vitro* antioxidant activity, *in vivo* anti-hyperglycemic effect of *Enterococcus* spp. on alloxan-induced diabetic albino rats and its attachment to the intestinal surface.

METHODS

Antioxidant activity by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

Antioxidant activity was performed by DPPH radical scavenging assay. Overnight grown bacterial cultures were centrifuged at 5000 rpm for 15 min. Cell-free the supernatant was added with a freshly prepared solution of DPPH at 5 mg/100 ml ethanol. Control was prepared using ethanol added to DPPH solution, mixed with dark, and incubated for 30 min. Absorbance was recorded at 517 nm using a spectrophotometer (Shimadzu, Japan). The percentage of radical scavenging activity was calculated according to the following equation [11].

$$\text{DPPH activity} = (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \times 100.$$

Preparation of bacterial suspension

The bacterial strains *E. faecium* and *E. hirae* used in this study were previously isolated from goat and sheep milk. These bacterial strains were separately cultured on MRS broth and grown at 37°C for 24 h. Then, cultures were harvested by centrifugation at 6000 rpm for 20 min. The

supernatant was decanted as the pellets of bacterial cells were washed twice with sterile phosphate buffer saline (PBS) and re-suspended in PBS to get the final concentration of 10^8 and 10^9cfu/ml, respectively, according to McFarland standards.

Test animals

Healthy albino rats (6–8 weeks old) were obtained from Lala Lajpat Rai University, Hisar (Haryana, India) and kept in the Animal House of the Department of Pharmaceutical Sciences, Gurukula Kangri (Deemed to be University), Haridwar (Animal House Reg. No.: 1324/a/10/CPCSEA). The Institutional Animal Ethical Committee approved the experimental protocol for use of laboratory animals. The rats were maintained in an air-conditioned room at 25°C in plastic cages with wood shaving as bedding and fed on commercial balanced sterilized diet pellets and water under standardized conditions.

Dose feeding procedure

The control groups received pure PBS saline. Each probiotic bacterial suspension of 10^8 cfu/ml and 10^9 cfu/ml, respectively, was administered orally once daily using PBS.

Induction of diabetes in albino rats

Diabetes was induced intraperitoneally in albino rats by injecting the freshly prepared alloxan dissolved in a normal saline solution of NaCl 0.9% pH 4 at a dose of 40 mg/kg body weight [12]. After 1 h of alloxan administration, animals were given feed *ad libitum*. Blood samples were collected for analysis using glucose test strips after 24 h of alloxan injection, from the vein at the tip of the tail. Blood glucose levels (BGL) were determined using a glucometer. Blood sugar level (250–350 mg/dl) was considered as a diabetic level.

Acute toxicity test

The rats were kept on fasting and provided with only water, thereafter *E. faecium* and *E. hirae* were administered orally by gastric tube in different gradual doses (10^7, 10^8, 10^9, and 10^{10} cfu/ml). Expression of any toxic symptoms and rat mortality was observed up to 72 h [13].

Experimental design

The animals were divided into seven groups and each group consisted of six animals. All animals were allowed to access feed and water. Group I was untreated rats as normal control. Group II was treated with alloxan monohydrate (at 40 mg/kg body weight) as diabetic control. Group III was treated with glibenclamide (at 50 mg/kg body weight) as a positive control. Groups IV and V received *E. faecium* (at doses of 10^8 cfu/ml and 10^9 cfu/ml); and Groups VI and VII were treated with *E. hirae* (at doses of 10^8 cfu/ml and 10^9 cfu/ml) as treatment groups. The blood glucose concentrations of the animals were measured at the beginning of the study and the same were repeated on the 3rd, 7th, 14th, and 21st days of treatment. All the animals were regularly observed for their general behavior. Changes in body weight were also measured.

Blood sampling and determination of BGL

Day 3 of induction was designated as day 1 for the administration of the test sample to diabetic rats. Fasting BGL were measured on days 3rd, 7th, 14th, and 21st of the administration period of test samples. Blood sampling was done by sterilizing the tail with 10% alcohol and then nipping the tail at the start of the experiment. After blood sampling, the tips of the tail were sterilized by swabbing with 70% ethanol. The BGL were determined using a glucometer.

Effect of probiotics bacteria on body weight

The effect of administration of probiotic strains to diabetic mice was determined by measuring the fasting BGL and changes in body weight. The body weight of each experimental set was measured with an electronic balance on 3rd, 7th, 14th, and 21st days.

***In vivo* study of intestine attachment of albino rats**

After approval granted from the Institutional Animal Ethical Committee (Animal House Reg. No. 1324/a/10/CPCSEA, Government of India), an experiment on albino rats was conducted in the Department of Pharmaceutical Sciences. The rats were divided into four groups and each group contains six animals. All albino rats (4–5 weeks old) were kept in light and dark cycle under standard conditions at a suitable temperature and fed with the standard pellet diet with water ad libitum. Consequent doses of *E. faecium* were prepared in saline having 10^9 cfu/ ml. These standard doses were given orally to rats after initiating the infection by different pathogens such as *Escherichia coli* and *Salmonella typhimurium*.

Excised rats intestine observed by scanning electron microscopy

After 7 days, after administrating the doses, albino rats were sacrificed by cervical dislocation. The small intestine of rats was removed gently. The segmented intestine was opened and washed with PBS. Washed intestine segments were fixed in 4% glutaraldehyde for 60–70 min and dehydrated using different concentrations of alcohol. Finally, the fixed and dehydrated samples were mounted on aluminum stubs further coated with gold-palladium. Attachment of *E. faecium* on the intestinal surface was observed by scanning electron microscope at 30 Kv in a LEO 485 VPSEM (ZEISS, USA). Photomicrographs were taken by the same microscope.

Statistical analysis

The data were analyzed statistically using Microsoft Excel 2010 and represented as a mean of triplicate±standard deviation. One-way analysis of variance (ANOVA) was employed followed by t-test: Two sample assuming equal variances. The values were considered significant level of $p<0.05$.

RESULTS

Antioxidant activity

The antioxidant activity of LAB strains plays an important role in the protection from free radicals. The antioxidant activity of *E. faecium* and *E. hirae* was measured by DPPH radical scavenging. The DPPH radical scavenging activity of *E. faecium* was 53.86% and 65.47% at 10^8 cfu/ml and 10^9 cfu/ml, which was higher than those of *E. hirae* showing 51.78% and 59.80% at 10^8 cfu/ml and 10^9 cfu/ml, respectively. *E. faecium* posed better antioxidant effects on the basis of radical scavenging activity (Fig. 1).

Antidiabetic activity of *E. faecium* and *E. hirae*

Glibenclamide administered to experimental animals resulted in a significant ($p<0.05$) rise in BGL. Before and after treatment with the test drug in glibenclamide-induced diabetic animals, changes in body weights and fasting BGL were determined. Fasting BGL in untreated diabetic rats were significantly higher (305.83 mg/dl) and the body weights were lower (89.50 g) than that of the normal rats. Diabetic animals treated with probiotic bacterial strains showed significant

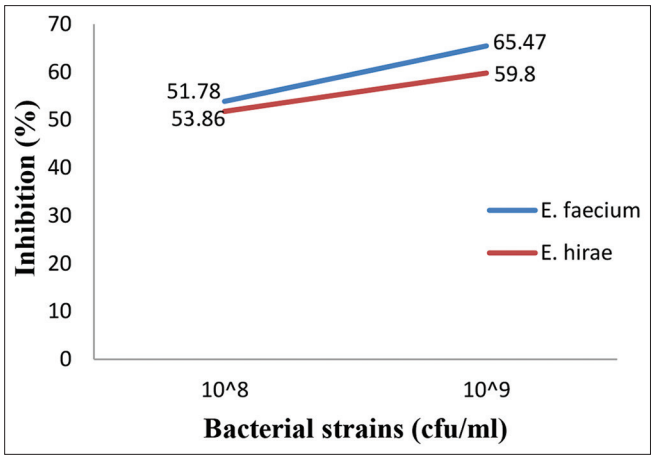

Fig. 1: Antioxidant activities of bacterial strains *Enterococcus faecium* and *Enterococcus hirae*

(p<0.05) lowering of BGL and a rise in body weights. Treatment of probiotic bacteria improved body weight and decreased BGL in alloxan-induced rats.

Acute toxicity assay

Cell suspensions of *E. faecium* and *E. hirae* were safe up to the highest dose of 10^{10} cfu/ml. The animal's behavior was observed for the first 4 h followed by an interval of every 6 h up to 72 h. Any physical sign of toxicity was not shown in albino rats by the bacterial cultures during the experiment and no mortalities were rewarded.

Effect of probiotic strains on BGL

Probiotic bacteria significantly (p<0.01) resulted in a dose-dependent reduction of BGL in alloxan-induced diabetic rats. Alloxanized rats of Group II (negative control) suffered from hyperglycemia as they did not receive any drug, whereas alloxanized rats of Group III (positive control) treated with the reference antidiabetic drug glibenclamide showed a significant reduction in BGL to the required standard BGL on the 14th day and the BGLs (116.50 mg/dL) were completely controlled under 21st day. The rats of Groups IV and V treated with *E. faecium* at doses of 10^8 cfu/ml and 10^9 cfu/ml, respectively, showed the decreases in BGL to 165.83 mg/dL and 143.66 mg/dL, respectively, which was close to the positive control group. However, Groups VI and VII rats treated with *E. hirae* at doses of 10^8 cfu/ml and 10^9 cfu/ml, respectively, and also showed the BGL of 173.16 mg/dL and 156.33 mg/dL, respectively, on 21st day. Positive control glibenclamide showed BGL normalized 116.50 mg/dL on 21st day, whereas *E. faecium* at the dose of 10^9 cfu/ml treated rats attained normal BGL of 143.66 mg/dL on the 21st day (Table 1).

Effect of probiotic strains on body weight of animals

There was a simultaneous increase in the body weight (139.16–148.16 g) of normal control rats during the experimental period 0, 3rd, 7th, 14th, and 21st days. All groups displayed the same bodyweight on 0 days, but the treated groups showed different body weight after the 3rd day. Group II diabetic control had the body weight (146.50 g) same as control groups for 0 days, but the diabetic group showed a decrease in body weight (137.66–89.50 g) the 3rd day. Group III (glibenclamide drug) exhibited the increasing body weight (118.66–136.66 g) continuously during the experiment. Groups IV and V treated with *E. faecium* showed ascended body weight (111.83–126.66 g at dose of 10^8 cfu/ml and 114.83–132.33 g at dose of 10^9 cfu/ml). Groups VI and VII treated with *E. hirae* ascended the body weight (109.33–124.83 g at dose of 10^8 cfu/ml and 112.16–129.33 g at dose of 10^9 cfu/ml) on 3rd, 7th, 14th, and 21st days and showed similarity with the control group. However, *E. faecium* at doses of 10^9 cfu/ml resulted in an increase in the bodyweight of rats as compared to positive control (Table 2).

Scanning electron microscopy of excised rat intestine

The presence of a large number of cells of *Escherichia coli* and *Salmonella typhimurium* was observed under the scanning electron micrograph of the small intestine of rats. While the treated rats were displayed the coccus-shaped cells of *E. faecium*. These *E. faecium* bacterial cells were aggregated with the consortium of different pathogens (Fig. 2).

DISCUSSION

This study was carried out to investigate the antioxidant activity, intestinal attachment and *in vivo* antidiabetic effect of two probiotics *Enterococcus* spp. in diabetic animals containing alloxan. The antioxidant effects of *Enterococcus* spp. were observed by the DPPH radical scavenging method. An imbalance in a large number of ROS and low antioxidant capacity is known as oxidative stress. The increased generation of ROS causes damage to cells, tissues, and biomolecules. ROS generation usually leads to cellular damage through several mechanisms, such as oxidation, interference with nitric oxide, and modulation of detrimental intracellular signaling pathways [14].

Animals with diabetes, obesity, hyperglycemia, and cholesterol induced by alloxan exhibited similar disease symptoms as in humans. The BGLs increase 3–4 times in alloxan-induced diabetic rats as compared to a normal control group. The pancreatic tissues decrease utilization of glucose or produce glucose, lipid in an excess amount that causes hyperglycemia in diabetes mellitus [15]. Alloxan has been shown to induce free-radical production. The formation of ROS like free-radical reduces the β-cells and causes tissue injury. The action of alloxan-induced free-radical damage is especially susceptible by the pancreas [16].

Table 1: Effect of probiotic *E. faecium* and *E. hirae* strains on blood glucose levels in alloxan-induced albino rats

Groups	Dose	Blood glucose concentration (mg/dl)				
		0 day	3rd day	7th day	14th day	21st day
Control	Normal saline	81.16±1.06	82.33±1.10	84.83±2.26	85.16±1.34	85.16±1.34
Diabetic control	40 mg/kg	86.83±1.95	244.83±3.89	287.33±1.49	308.16±1.06	305.83±4.56
Glibenclamide	50 mg/kg	83.16±1.06	229.83±1.34*	217.16±2.40*	193.83±1.06*	116.50±1.70*
E. faecium	10^8 cfu/ml	85.66±1.59	236.66±1.10**	229.16±0.68*	218.33±0.74*	165.83±1.77*
E. faecium	10^9 cfu/ml	84.16±1.21	232.83±1.77*	221.83±1.34*	204.16±2.11*	143.66±1.10*
E. hirae	10^8 cfu/ml	86.16±1.34	238.83±1.46***	231.83±1.57*	222.83±1.34*	173.16±1.06*
E. hirae	10^9 cfu/ml	85.16±1.21	235.66±0.74**	226.66±0.74*	211.50±1.70*	156.33±0.74*

The values are mean ± SD of six rats in each group. One-way ANOVA followed by t-test: Two sample assuming equal variances; *** $P < 0.05$, ** $P < 0.01$, * $P < 0.001$ as compared to diabetic control. *E. faecium*: *Enterococcus faecium*, *E. hirae*: *Enterococcus hirae*

Table 2: Effect of probiotic *E. faecium* and *E. hirae* strains on body weight of alloxan-induced albino rats

Groups	Dose	Body weight (g)				
		0 day	3rd day	7th day	14th day	21st day
Control	Normal saline	139.33±1.24	143.16±2.26	144.16±1.57	146.16±1.34	148.16±0.89
Diabetic control	40 mg/kg	146.50±1.50	137.66±0.74	127.50±3.14	91.83±1.83	89.50±1.11
Glibenclamide	50 mg/kg	139.16±1.06	118.66±1.10**	121.83±1.34***	127.50±0.95*	136.66±1.10*
E. faecium	10^8 cfu/ml	140.83±1.95	111.83±1.06*	114.16±0.68*	118.33±0.74*	126.66±3.94*
E. faecium	10^9 cfu/ml	142.16±1.34	114.83±1.34*	117.83±0.68*	122.33±1.10*	132.33±1.10*
E. hirae	10^8 cfu/ml	138.66±1.49	109.33±0.94*	112.16±1.34*	116.83±1.06*	124.83±2.40*
E. hirae	10^9 cfu/ml	141.33±1.24	112.16±1.06*	115.16±0.89*	120.66±2.80*	129.33±0.94*

*The values are mean±SD of six rats in each group. One-way ANOVA followed by t-test: Two sample assuming equal variances; *** $P<0.05$, ** $P<0.01$, * $P<0.001$ as compared to diabetic control. *E. faecium*: *Enterococcus faecium*, *E. hirae*: *Enterococcus hirae*

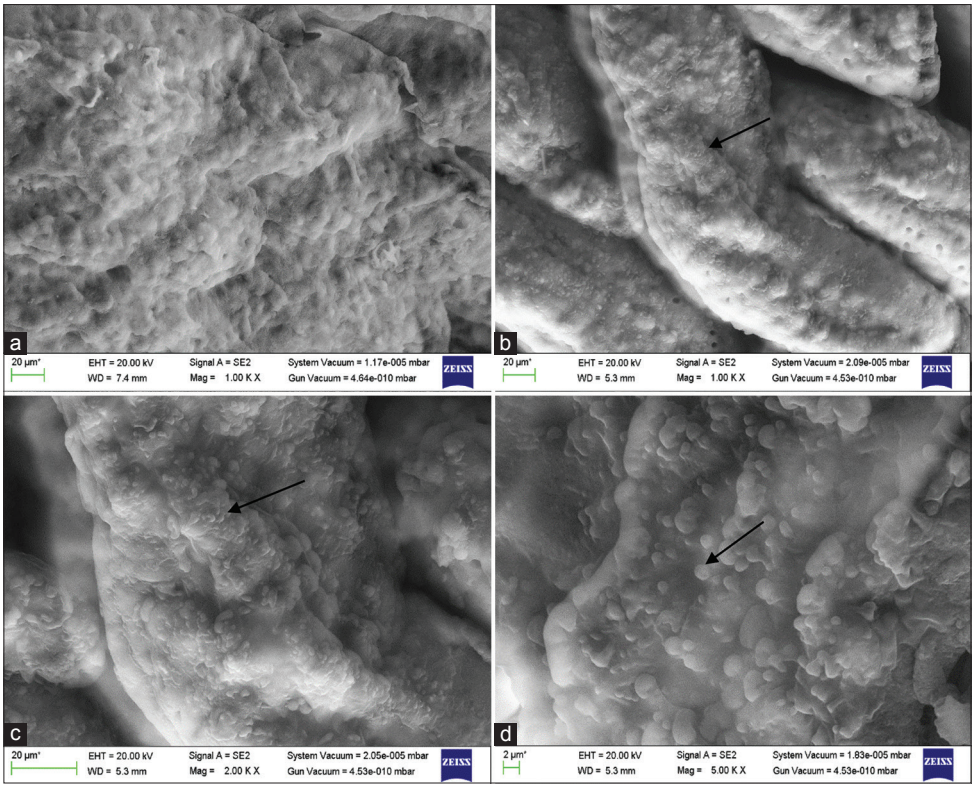

Fig. 2: Intestinal attachment of probiotic *Enterococcus faecium;* (a) control, group; (b) rats infected by consortium of different pathogens; (c) probiotic *E. faecium* aggregates with pathogenic bacterial cells; (d) coccus-shaped *E. faecium* in the intestine of the treated rats

Our results displayed that BGL reduced by the glibenclamide in hyperglycemias animals. In this condition, the state of diabetes is not severe. At the end of the experimental period, a significant reduction in BGL was recorded in alloxan-induced diabetes animals treated by probiotic bacteria as compared with diabetic control groups. Induction of diabetes with alloxan is associated with the characteristic loss of body weight, due to increased muscle wasting in diabetes. Moreover, the diabetic rats treated with the probiotic bacteria gained the body weight as compared with the diabetic control group. A marked increase was observed in serum triglycerides, cholesterol, and HDL in untreated diabetic rats. A similar finding has also been reported by Nikkila and Kekki [17]. Diabetic rats treated with the probiotic bacteria showed a significant decrease in cholesterol and triglycerides and an increase in HDL as compared with diabetic control. During diabetes, glycogen syntheses in the rat liver and skeletal muscles were reduced. Therefore, alloxan may produce oxygen radicals in the body. This causes pancreatic injury and could be responsible for increased blood glucose [18]. The antidiabetic effects of the *E. faecium* (doses of 10^9 cfu/ml) were almost similar to glibenclamide. This is a possible mechanism by which the probiotic bacteria have antidiabetic action due to potentiation of pancreatic secretion of insulin from existing residual β-cell of islets or improved the transport of blood glucose to peripheral [19].

In this study, the accumulative action of the probiotic bacterial strains might have a progressive reduction in the BGL of alloxan-diabetic rats during the treatment period and also associated with an increase in the blood insulin levels. In alloxan-diabetic rats, elevation in blood insulin by the probiotic bacterial treatment could be because of the high antioxidant activities exhibited by probiotic bacteria that encourage the protection of the functional β-cells from further deterioration or the β-cells regenerate so that they remain active and produce insulin. The probiotics have a significant role in the treatment or prevention of various chronic diseases, including diabetes by lowering the BGL as also confirmed in this study.

Adhesion of pathogenic bacteria to the surface of mucus is considered to be an initial step of intestinal infections. The adhesions of pathogens recognize specific mucosal receptors, which is mediated by bacterial adhesions. Adhesion may be inhibited with specific adhesion analogs by blocking the receptor. Therefore, inhibition of adhesion may prevent the colonization of the intestine by the pathogen and thereby prevent the infection. Some probiotic bacteria with helpful health effects have been found to adhere to the intestinal mucosa. Consequently, adherent probiotic bacteria could prevent the subsequent attachment of pathogens, and referred to as "competitive exclusion." In the intestine, epithelial cells are covered with a mucus layer protecting the epithelial cells from physical and chemical damage as well as from pathogenic bacteria [20]. The mucus layer is most likely the first place of contact between the host and the pathogen.

CONCLUSION

The present study demonstrates that the antioxidant and antidiabetic activity of probiotic bacterial strains, *E. faecium* and *E. hirae*. These bacteria exhibited the greatest antioxidant activity estimated through the scavenging of free radicals such as DPPH. Due to its potent antioxidant properties, free-radical scavenging in probiotic bacterial strains could be responsible for its antidiabetic effect. These probiotic bacterial strains reduced the glucose level and body weight gain in alloxan-induced diabetic rats. In addition, *E. faecium* may have modified the intestinal conditions by inhibiting the pathogens and pathogens adhering to the intestinal cell.

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AUTHORS CONTRIBUTIONS

Both the authors mutually contributed in preparation of research paper.

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CONFLICT OF INTEREST

There is no conflict of interest.

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