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Original research article

The hypoglycemic potential of endophytic *Lactobacillus kunkeei*, isolated from Dandelion flowers in rats

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ABSTRACT

Blood glucose level control is a widely used approach, as diabetes mellitus is increasing worldwide. Lactobacillales have recently attracted the greatest attention due to their diabetes alleviating effects. Therefore, the current study was designed with the objective to evaluate the antidiabetic potential of entophytic *Lactobacillus kunkei* isolated from local *Taraxacum kok-saghyz L.* in glucose-, adrenaline-, and alloxan-induced diabetic rat models. In this study, the *L.kunkeei* strain was isolated from local *Taraxacum kok-saghyz L.* (locality Tashkent, Uzbekistan). Blood glucose in glucose-, adrenaline-, alloxan-induced and MDA level in alloxan-induced diabetic rats under *L. kunkeei* pretreatment was evaluated. In the current study, blood glucose levels were significantly improved by *L. kunkeei* in glucose-, adrenaline- and alloxan-induced diabetic rats *in vivo*. In addition, the strain exhibited *in vitro* α-amylase inhibiting effects, as well as reduced oxidative stress in the liver and the pancreas of alloxan-induced diabetic rats. Based on these studies, it is believed that food supplements of specific lactic acid bacteria strains can be effective as a preventive strategy against metabolic disorders. However, further long-term studies are needed to validate the specific constituents and, thus, the mechanisms responsible for the hypoglycemia-reducing effect of *L. kunkeei*.

Keywords: lactic acid bacteria, glycemic control

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1. Introduction

Glycemic control is an effective long-term therapy for people with type II diabetes mellitus, reducing the risk of cardiovascular and neurological complications during the onset of the disease.[1] Enzyme inhibitors are usually prescribed for diabetics to reduce postprandial hyperglycemia caused by the digestion of starch in the small intestine.[2] The overall effect of inhibition is to reduce the flow of glucose from complex dietary carbohydrates into the bloodstream, which reduces the postprandial effect of starch intake on blood glucose levels. At the same time, oxidative stress in diabetes mellitus is a vicious cycle associated with an increase in the sources of formation of free radicals, potentiation of the mechanism of their toxic action and changes in the activity of the antioxidant system resulting in tissue damage.[3] Thus, the potential search is for substances that not only normalize hyperglycemia, but also limit the formation of free radicals. Such substances were proven to be found in large quantities in plant substrates. However, over the past 10 years, more than 200 new biologically active compounds have also been isolated from endophytes, making them "alternative" valuable sources of the same compounds as the host plant, including polyphenols, alkaloids, flavonoids, fatty acids, and others.[4] At the same time, symptoms of hyperglycemia can develop quite quickly, so an immediate response is important to the slightest of their manifestations. The higher the blood sugar level and the longer it is maintained, the more dangerous the condition can be. The Taraxacum genus from the Asteraceae family is used in traditional medicine as food and in some countries as therapeutics for the control and treatment of T2D.[5] Uzbekistan possesses rich reserves of medicinal plants, including endemic ones, which have been used in traditional medicine for centuries. Regarding diabetes, dandelion roots, leaves and flowers have been used for a long time in traditional medicine as antidiabetic solutions. Recently, Amirsaidova et al. demonstrated that endophytic *Lactobacillus kunkeei*, isolated from Dandelion flowers (*Taraxacum officinale*), orally administered, and reduced blood glucose levels in hyperglycemic rats.[6] The current study was designed with an objective to evaluate the antidiabetic potential of *Lactobacillus kunkei* isolated from endemic *Taraxacum kok-saghyz L.* in glucose-, adrenaline- and alloxan-induced diabetic rat models.

2. Materials and Methods

2.1 Chemicals, reagents and bacteria

The strain *L. kunkeei* was isolated from dandelion flowers (*Taraxacum kok-saghyz L.*) grown in Uzbekistan (Tashkent locality) and kept as a frozen stock in the laboratory of "Microbiology and biotechnology of probiotics" of the Institute of Microbiology, Uzbekistan Academy of Sciences (Tashkent, Uzbekistan). The strain was recovered from the frozen stock by culturing in MRS broth (HiMedia, India) and incubated at 30°C for 24 h,[7] washed and resuspended in sterile saline, the turbidity adjusted to 10^8-10^9 CFU/mL.

2.2 Malondialdehyde (MDA) assay

Thiobarbituric Acid Reactive Substances (TBARS) levels, measured as an index of MDA (malondialdehyde) production and hence lipid peroxidation, were assessed in the tissues by the basic protocol of Heath and Packer.[8] In brief, supernatants from liver and pancreas tissue homogenates (1 ml) of alloxan-induced rats were added separately to test tubes containing 4 ml of TCA (trichloroacetic acid) 20%, containing TBA (thiobarbituric acid) 0.5%, and the reaction mixture was heated at 95°C for 30 min and, after cooling, centrifuged at 10,000 g for 10 min; the MDA/TBA complex was measured spectrophotometrically at 532 nm.

2.3 Determination of the inhibitory activity of samples against α-amylase *in vitro*

The inhibitory activity of the *L. kunkeei* samples on α-amylase was determined in pancreas homogenates of 5 mature rats according to A.M. Ugolev.[9] The experimental protocols were approved by the Institute's Animal Ethics Committee based on the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes.[10] The animals were decapitated by a guillotine, and the pancreas was removed from the abdominal cavity, cleared of adipose tissue, weighed and homogenized in a glass homogenizer with a Teflon pestle at 400 rpm for one minute. The resulting homogenate was centrifuged at 3,000 rpm for 15 min. For experiments, the supernatant was taken and stored on ice. Protein content in tissues was determined by the Lowry method modified by Hartree.[11]

2.4 Animal experiments

Healthy adult male outbred rats (200 ± 20 g) were provided by the Pharmacological Laboratory (Institute of Bioorganic Chemistry, Uzbekistan Academy of Sciences, Tashkent, Uzbekistan). The experiments were carried out according to the guidelines of the Institute's Animal Ethics Committee based on the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes.[10] Animals were kept in a specific pathogen-free habitat and were divided into groups, housed in polypropylene cages and fed laboratory chow and water *ad libitum* during the whole experiment.

2.5 Acute toxicity test

The acute toxicity test was performed on 30 mature white outbred rats (male, 200 ± 20 g) according to Litchfield JT et al.[12] with some modifications. Animals were randomly divided into 6 groups, 5 animals per group. Group I rats served as the control group and received distilled water throughout the experiment, while groups II-VI rats were administered orally with 4×10^8, 5×10^8, 6×10^8, 8×10^8 and 10×10^8 CFU/rat of the *L. kunkeei* sample, respectively. The animals were monitored for signs of toxicity hourly during the first day of the experiment in the laboratory (general health status, possible convulsions, death) and then, daily for 2 weeks under vivarium conditions.

2.6 Oral glucose tolerance test

The acute hypoglycemic activity was determined by an oral glucose tolerance test on 14 mature white outbred rats (male, 200 ± 20 g) as described elsewhere.[13,14] Rats were weighed before the experiment, and the initial blood glucose level was determined as a control. Blood was taken from the tail of rats, under conditions that prevented excessive excitement of animals. Sixty minutes prior to glucose administration, the *L. kunkeei* samples (10×10^8 CFU/mL/rat) were orally introduced to experimental animals using a gastric catheter. Acute hyperglycemia was induced by a single intragastric administration of a hypertonic glucose solution at a dose of 5000 mg/kg. Blood glucose levels were determined after 1 hour using the Cypress Diagnostic test kit (Germany). The negative control group (intact group, 4 animals) contained animals that were administered neither *L. kunkeei* nor glucose solution.

2.7 Alloxan-induced hyperglycemia

The alloxan-induced hyperglycemia was determined in 15 mature white outbred rats (male, 200 ± 20 g) according to to[15] with minor modifications. Rats were weighed before the experiment and divided into 3 groups (5 rats per group). The intact group contained animals that were administered neither *L. kunkeei* nor alloxan. The control group contained alloxan-induced hyperglycemic rats, the experimental group contained both *L. kunkeei* and alloxan- administered animals. Experiments were carried out as follows: 60 min before hyperglycemia induction, the *L. kunkeei* samples (10×10^8 CFU/mL/rat) were orally introduced to experimental animals using a gastric catheter. Hyperglycemia was induced by intraperitoneal injection of alloxan in a single dose of 100 mg/kg. Blood was

taken from the tail of rats under conditions that prevented excessive excitement of animals. Blood glucose levels were determined after 1 hour using the Cypress Diagnostic test kit (Germany).

2.8 Adrenaline-induced hyperglycemia

The adrenaline-induced hyperglycemia was determined on 15 mature white outbred rats (male, 200 ± 20 g).[16] Rats were weighed before the experiment, and the initial blood sugar level was determined as a control. Blood was taken from the tail of rats under conditions that prevented excessive excitement of animals. Sixty minutes before hyperglycemia induction, the *L. kunkeei* samples (10×10^8 CFU/mL/ rat) were orally introduced to experimental animals using a gastric catheter. Hyperglycemia was induced by intraperitoneal injection of adrenaline in a single dose of 50 mg/kg. The blood glucose level was determined after 1 hour using the Cypress Diagnostic test kit (Germany).

2.9 Statistical analysis

A t-test was used for statistical analysis. Data were expressed as scatter plots using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, California, USA). The results were considered statistically significant at $P<0.05$.

3. Results

3.1 Acute oral toxicity test

Short-term exposure of experimental animals to $4–6\times10^8$ CFU/rat of *L. kunkeei* after 5–10 min provoked 50% excessive grooming, increase of respiratory frequency up to 50%, and spatial patterning showed huddled up behavior that was not monitored in the control group. After 2–3 hours, the animals recovered and returned to their normal behavioral patterns. No mortality was observed. Also, no treatment-related toxic symptoms or mortality was observed after oral administration of the tested *L. kunkeei* at a dose of $8–10\times10^8$ CFU/mL/rat. However, there were signs of sedation that manifested in 80% excessive grooming, huddled up behavior, and eye pupil shrinking (100%) compared to the control group that returned to the typical pattern in 4–5 hours. On the 14th day after oral *L. kunkeei* administration, rats appeared healthy and unstressed. The parameters observed for acute toxicity after *L. kunkeei* administration are presented in Table 1.

3.2 Oral glucose tolerance

The blood glucose level was measured before experimental manipulations to get a baseline which was 3.5±0.3 mM/L ($P<0.001$) (intact group). Short-term oral glucose-induced mild hyperglycemia was monitored in the control group (8.7±0.5 mM/L, $P<0.001$) compared to the untreated group. Fig. 1 depicts the antihyperglycemic effect of 10×10^8 CFU/mL/rat of *L. kunkeei* administered 1 hour prior to the oral hyperglycemic agent (glucose) (experimental group). One hour after hyperglycemic stress the blood glucose concentration was reduced by up to 33.3 % in *L.kunkeei*-treated group.

Table 1. Acute oral toxicity test of *L. kunkeei* lactic acid bacteria in rats.

Dose (CFU/mL)	4×10^8	5×10^8	6×10^8	8×10^8	10×10^8
Signs of toxicity	none	none	none	none	none
Survival	6/6	6/6	6/6	6/6	6/6

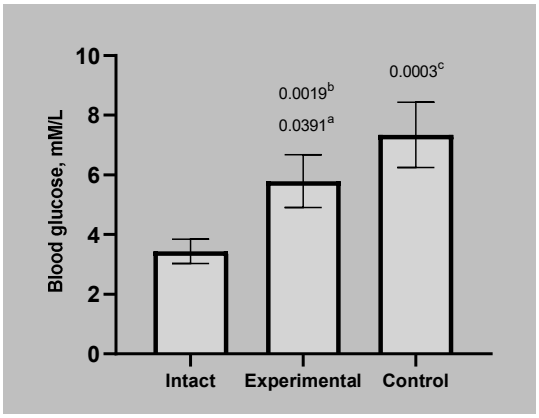

Fig.1. The effect of oral administration of *L. kunkeei* on blood glucose levels in rats (n = 4-5). Note: a - in comparison to control group, b - in comparison to intact group, c - in comparison to intact group. Intact group - untreated animals, control group - glucose-induced hyperglycemic animals, experimental group - *L. kunkeei* administered animals.

3.3 α-amylase inhibition activity

The percentage inhibition of α-amylase by *L. kunkeei* was studied in the concentration range of 2×10^8–10×10^8 CFU/mg protein. The percentage inhibition of α-amylase by *L. kunkeei* was linear (y = 2.227x-2.745, R^2 = 0.94), in an appreciable dose-dependent manner. The maximum dosage 10×10^8 CFU/mg protein caused up to 25% inhibition (Fig. 2). Assuming that higher concentrations of bacteria would inhibit the enzyme with linear dependence, the IC_{50} calculated from curve fitting was 23.8×10^8 CFU/mg protein. In order to elucidate the possible modes of action of *L. kunkeei*, it is necessary to investigate these findings on a suitable animal model.

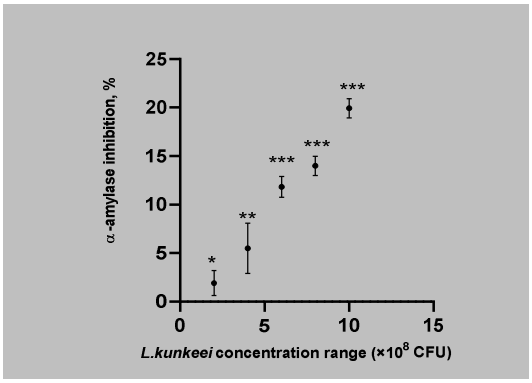

Fig.2. α-amylase inhibitory activity of *L. kunkeei* (n = 5).

3.4 Adrenaline-induced hyperglycemia inhibition activity

In the adrenaline-induced hyperglycemia model, adrenaline stimulates glucose release from glycogen stores in the liver into the blood stream.[16] Adrenaline infusion increased blood mean glucose concentration from 3.5±0.3 mM/l to 10.1±1.0 mM/L ($P\leq 0.05$), consistent with previous observations[32]. Evaluation of the effect of *L. kunkeei* in adrenaline-induced hyperglycemia revealed that this strain did cause appreciable reduction of the hyperglycemic response. In intact animals, *L. kunkeei* did not cause a rise in glucose production. As shown in Fig.3, administration of physiological saline did not impact the basal blood glucose in normal rats after 2 hours of monitoring. In contrast, *L. kunkeei* abolished approximately 50% of the adrenaline-induced hyperglycemia; the average blood sugar concentration after adrenaline infusion was 10.1±1.0 mM/L ($P\leq 0.05$) in the control animals and 4.1±0.35 mM/L ($P\leq 0.05$) after *L. kunkeei* treatment.

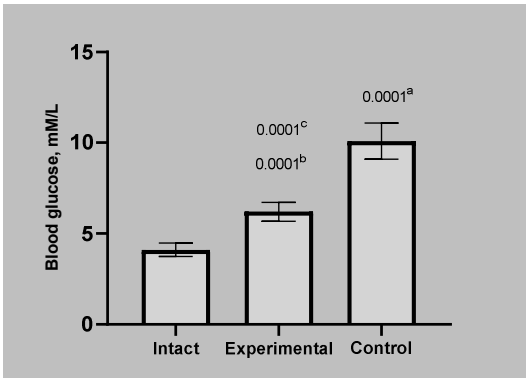

Fig.3. Effect of oral administration of *L. kunkeei* on the blood glucose level in adrenaline-induced hyperglycemic rats. Note: a - in comparison to intact group, b - differences between the untreated and treated animals, c - in comparison to intact group. Intact group - untreated animals, control group - adrenaline-induced hyperglycemic animals, experimental group - *L. kunkeei* administered animals**.**

3.5 Alloxane-induced hyperglycemia inhibition activity

Average blood glucose levels are presented in Fig. 4. In the diabetes-induced groups, blood glucose concentration levels declined significantly ($P < 0.0001$) in comparison to the final concentrations in the control group. Blood glucose levels decreased significantly ($P < 0.0001$) in the diabetic group pre-treated with *L. kunkeei*, compared to the intact group of animals.

3.6 Lipid peroxidation inhibition activity

The level of MDA was determined with the aim of observing the oxidative damage caused to lipids (Fig.5). The MDA concentration in the alloxan-induced diabetic group was significantly ($P < 0.0001$) higher when compared to the intact group. The MDA concentrations in the diabetic groups, pre-treated with *L. kunkeei* were significantly ($P < 0.0001$) compared to the diabetic group without *L. kunkeei* pre-treatment.

Fig.4. Effect of the oral administration of *L. kunkeei* on the oral glucose tolerance in alloxan-induced hyperglycemic rats. Note: a - in comparison to intact group, b - differences between the untreated and treated animals, c – in comparison to intact group. Intact group - untreated animals, control group - alloxan-induced hyperglycemic animals, experimental group - *L. kunkeei*-administered animals.

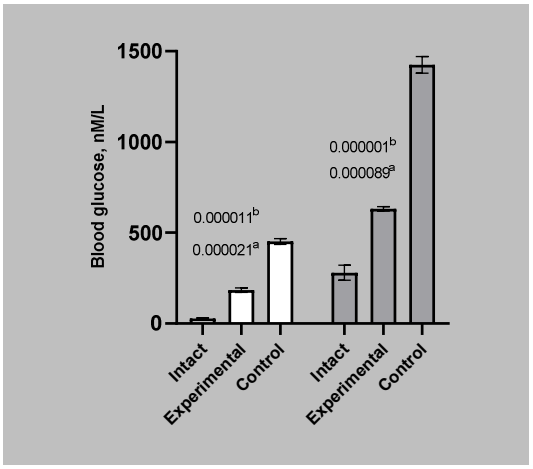

Fig.5. *In vitro* effects of *L. kunkeei* treatment on MDA concentration in liver (white columns) and pancreas (gray columns) of alloxan-induced diabetic rats. Note: a - in comparison to intact group, b - in comparison to alloxan-induced group. Intact group - untreated animals, control group - alloxane-induced hyperglycemic animals, experimental group - *L. kunkeei* administered animals.

4. Discussion

In the present work *L. kunkeei* was isolated from the endemic *Taraxacum kok-saghyz* L. and its hypoglycemic effect was

proven. Oral consumption of *L. kunkeei* titred to 10^8-10^9 CFU reduced blood glucose levels in glucose-, adrenaline- and alloxane-induced hyperglycemic experimental rats within 1 hour. The rapid glucose levels rise observed in control groups is usually associated with the absorption of carbohydrates in the intestine. Thus glucose levels decreased by L. kunkeei may result from suppression of glucose absorption from the intestine. Findings from similar studies[17,18] provide circumstantial evidence that gut microbiota, namely lactic acid bacteria, might be involved in the improvement of glucose homeostasis.

As does the commonly prescribed acarbose, *L. kunkeei* culture broth exhibited α-amylase inhibition and reduced oxidative stress caused by alloxane. The amylase-inhibiting activity of various lactic acid bacterial strains has been widely examined and remains without doubt. Thus, α-amylase inhibition was shown for *Lactobacillus casei* Zhang and *Lactobacillus casei* SY13,[19] *Lactiplantibacillus plantarum* and *Lacticaseibacillus paracasei* strains,[20] etc. The hypoglycemic action observed in this paper might be due to this strain's characteristics, such as the ability to degrade macromolecules, i.e. indigestible polysaccharides and proteins.[21] On the other hand, diabetes-accelerated complications are caused mainly through mitochondrial reactive oxygen species overproduction.[22] Several mechanisms mediating the antioxidant ability of lactic acid bacteria have been proposed. Abubakr et al.[23] demonstrated the antioxidant activity of *L. plantarum* and *L. pentosus* isolated from grapes, banana and apples mediated by peptides in whey from skimmed milk. Kuo et al. observed high DPPH-inhibiting activity (93.2%) by *L. plantarum* BCRC 16000 and 11697 which correlated with a high content of phenolic compounds.[24] It could also be important how the experiments are designed to provide an adequate values of the results received. Thus our findings are for the pre-treatment of experimental animals by the lactic acid bacteria suggesting the effects are those of a preventive measure. On the contrary, Mazloom et al. revealed a non-significant inhibition of MDA content, as well as insulin resistance, after consumption of lactic acid bacteria by type 2 diabetic patients who have had diabetes for less than 15 years.[25] Considering the results of this study in terms of global trends, the beneficial effect of specific *L. kunkeei* isolates from endemic *Taraxacum kok-saghyz L.*, i.e. the reduction of glucose and oxidative stress might be an outcome of the production of the same bioactive compounds as those of its host plant. Therefore, the present study provides a promising microbial source of high-value products with significant therapeutic activities against hyperglycemia and oxidative stress, necessitating further studies to explore the potential applications of epiphytic lactic acid bacteria.

5. Conclusion

The blood glucose level is a reflection of the state of two constantly changing processes under constant control of insulin: glucose utilization by tissues and glucose entry into the bloodstream. One important therapeutic approach for diabetes is to decrease hyperglycemia. The present study, therefore, aimed at exploring the potential of *L. kunkeei* isolated from *Taraxacum kok-saghyz L.* as a prospective anti-hyperglycemic agent. Based on our study, we believe that food supplements of specific lactic acid bacterial strains can be effective as a preventive strategy against metabolic disorders. However, long-term studies are needed to validate the specific constituents and mechanisms of *L. kunkeei* responsible for reducing hyperglycemia.

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Conflicts of Interest

The authors declare no conflict of interest.

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