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Research Article

# Effect of Supplementation of Probiotic and their DNA alone or in Combination with Glyburide on Hyperglycemic Swiss Albino Mice

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**ABSTRACT**

The recent increase in the incidence of diabetes raises a need to find new alternative therapies to control it. Probiotics reduces the blood glucose concentrations .But recently bacterial DNA is also being explored as an immune enhancer. Present study was conducted to compare the *in vivo* antidiabetic capacity of three probiotic strains as live bacteria with their genomic DNA. Probiotic bacteria's ($10^9$ cells $ml^{-1}$) were administered orally to swiss albino mice whereas their extracted DNA's (75 µg $mL^{-1}$) were injected into the tibialis anterior muscle. Experiment involved the acute study (24 h) and sub-acute study (28 days). Standard drug Glyburide (10 mg/kg b.wt) was given alone and also in combination with probiotic and with their genomic DNA. Oral glucose tolerance test (OGTT) in normal and diabetic mice was also tested *in vivo.* The blood samples so collected were analyzed for blood glucose levels. The combination of standard drug (glyburide) and DNA of probiotics exerted an antihyperglycemic effect and showed better reduction in glucose levels than glyburide alone. However, probiotic bacteria showed only 2.71 % decrease in glucose level in comparison to DNA which showed 16.7 % decrease. It is concluded that genomic DNA of probiotics should be exploited as a potent antihyperglycemic biotherapeutic agent.

**Keywords**: *Lactobacillus; Bifidobacterium;* Antihyperglycemic activity; Bacterial DNA

## 1. INTRODUCTION

Diabetes causes metabolic disturbances which underlie the action of many systems including some higher functions of the brain such as learning and memory. Plenty of evidence supports the effects of probiotics on the function of many systems including the nervous[1], cardiovascular[2] and immune [3].Probiotic treatment reduces the blood glucose level and acts as natural hyperglycemic agents[2,4].Recently, some bacterial cell components such as peptidoglycans, lipoteichoic acid, secrete soluble substances [5,6] and genomic DNA[7] reportedly play role in immunomodulation responses but primary component is yet to be identified.

The immunostimulatory effect by bacterial DNA were defined to be dependent upon short sequences of CpG dinucleotides which differ from that found in eukaryotic DNA[8,9]. Only in bacterial DNA, unmethylated CpG motifs can be found [10]. In eukaryotic DNA, CpG-containing sequences occur at a much lower frequency than in bacterial DNA [11,12] and they appear to be underrepresented in eukaryotic genomes; a phenomenon known as "CpG suppression" and when it is present, the cytosine is methylated [13],which prevents their immune stimulatory effects [14,8].

It has been evidenced that Bacterial DNA and immunostimulatory CpG-ODNs activate Antigen Presenting Cells (APCs) such as macrophages and dendritic cells. Cell activation

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occurs upon DNA endosomal uptake, resulting within minutes in activation of the Stress Kinase pathway and NF-kB. As a consequence, APCs produce cytokines including IL-12, IL-6 and IL-1 and upregulate coreceptor molecules [15].

The purpose of current study was to compare *in vivo*, anti diabetic activity of probiotic viable bacteria's with their isolated genomic DNA.

## 2. MATERIALS AND METHODS

**2.1 Bacterial strain and culture condition:** The strain of *Lactobacillus delbrueckii* 405 (LB 405) , *Lactobacillus brevis 403* (LB 403), *Bifidobacterium bifidium* BD4 234 (Bif 234) was procured from National Dairy Research Institute, Karnal, Haryana. The cultures so obtained were given two revival cycles in de Man–Rogosa–Sharpe broth (MRS broth) at 37 °C. Bacterial cultures were grown and maintained for further use. For genomic DNA preparation, cells were grown in the corresponding medium containing 1 to 1.5 % glycine to facilitate cell lysis[16].

**2.2 Preparation of genomic DNA of bacterial strain:** Genomic DNA was isolated and purified with several modifications [16].Briefly, an overnight culture (1.5 ml) was pelleted at 14000 rev $min^{-1}$ (microcentrifuge) 25°C for 5 minutes and resuspended in 500µL EDTA (50$mM^{-1}$). 100 µL of 30$mgml^{-1}$ Lyosozyme was added to cell suspension and incubated for 60 minutes at 37°C. Cell lysis was achieved using NaOH/SDS solution (pH 12.5) and incubation 20 min at 37°C followed by 10 min incubation on ice. Protein removal was carried out with phenol followed by chloroform: isoamyl alcohol (24:1) extraction. DNA was precipitated by addition of isopropanol and washed with 70% ethanol to remove residual contamination. DNA was then resuspended in 20-30 µL of TE (Tris 10mM, EDTA 1mM pH 8.0).The concentration and purity of DNA were analyzed spectrophotometricaly (Shimadzu, UV-1650 PC spectrometer) by measuring $OD_{260}/OD_{280}$.Only the DNA with $OD_{260}/OD_{280}$ ratio ranging between 1.8 and 2.0 respectively was used. The quality of DNA was further analyzed on 1 % agarose gel (100V for 20-40 min) containing 0.5 $\mu gm^{-1}$ ethidium bromide. The endotoxin level in the DNA preparation were <0.001 ng$\mu g^{-1}$ of DNA according to Limulius amebocyte lysate assay.

**2.3. Mice:** Swiss albino male mice (18-22gm) maintained on standard laboratory diet (Kisan Feeds Ltd., Mumbai, India) and water *ad libitum* were employed in the present study. The animals were divided into respective groups each of minimum six animals, housed individually in the departmental animal house and were exposed to 12 hr cycle of light and dark. The experimental protocol was approved by Institutional Animal Ethical Committee (Registration No: 107/99/CP-CSEA-2010-40) were carried out as per the guidelines of committee for Purpose of Control and Supervision of Experimental on Animals (CPCSEA) Ministry of Environment and Forest, Government of India.

**2.4 Induction of experimental diabetes :** Experimental diabetes was induced by intraperitoneal (i.p.) administration of glyburide in mice which had been subjected to overnight fasting [17] .Total dose of glyburide (150 mg/kg b.wt) was administered in three injections at intervals of 24 h (50 mg/kg b.wt each time). After 72 h, animals showing blood glucose level above 200 mg/dl (diabetic) were selected for study.

**2.5 Collection of blood and determination of blood glucose:** Blood of control and experimental mice was collected from orbital sinus puncture using a heparinized capillary glass tube. The blood samples so collected were analyzed for blood glucose levels by glucose estimation kit (Bayer health care LLC, Ireland). Blood glucose levels were estimated according to the method of Dunn and McLetchie [18].

**2.6 Experimental design:** Diabetic swiss albino mice of either sex were divided into six groups (n = 6).

Group I: **Untreated control** i.e. mice fed basal feed.

Group II: **Drug control** (Glyburide,10 mg/kg b.wt)

Group III: **LB 405** i.e. Hyperglycemic mice dosed with LB 405 $10^9$cells $day^{-1}$ $mouse^{-1}$ as oral dose

Group IV: **DNA LB 405** i.e.Hyperglycemic mice dosed with DNA of LB 405 in left tibialis anterior muscle [19] at the rate of 75µg $mL^{-1}$/mouse

Group V: **LB 405 + Drug** i.e. Hyperglycemic mice dosed with glyburide (10 mg/kg b.wt) and LB 405 ($10^9$cells $day^{-1}$ $mouse^{-1}$).

Group VI: **DNA LB 405 + Drug** i.e. Hyperglycemic mice dosed with glyburide (10 mg/kg b.wt) and DNA of LB 405 (75µg $mL^{-1}$ $mouse^{-1}$).

Experiment involved the acute and sub-acute study. The **acute study** involved the estimation of blood glucose levels at 0, 2, 4, 6, and 24 h after administration of LB 405, DNA LB 405, drug and combinations with all the doses given at one time.

The **sub-acute study** involved the repeated administration of drug for 28 days at a prefixed time and blood glucose levels were estimated on days 7, 14, 21, and 28. The data were represented as mean blood glucose level and standard error mean (SEM).

**2.7. Oral glucose tolerance test (OGTT) in normal and diabetic mice:** Prior to commencement of experiment, the animals were fasted overnight. After 30 min of doses administration, animals were loaded with D-glucose solution (2.5 mg/kg) and blood glucose levels were monitored at 0, 30, 60, and 120 min after glucose loading.

Non-diabetic and diabetic mice were divided into six groups (n = 6)

Group I: **Untreated control** i.e. mice fed basal feed.

Group II: **Drug control** (**Glyburide**,10 mg/kg b.wt)

Group III: **LB 405** i.e. Hyperglycemic mice dosed with LB 405 ($10^9$cells $day^{-1}$ $mouse^{-1}$) as oral dose

Group IV: **DNA LB 405** i.e. Hyperglycemic mice dosed with DNA of LB 405 in left tibialis anterior muscle [19] at the rate of 75µg $mL^{-1}$/mouse.

Group V: **LB 405 + Drug** i.e.Hyperglycemic mice dosed with glyburide (10 mg/kg b.wt) and LB 405 ($10^9$cells $day^{-1}$ $mouse^{-1}$).

Group VI: **DNA LB 405 + Drug** i.e.Hyperglycemic mice dosed with glyburide (10 mg/kg b.wt) and DNA of LB 405 (75µg $mL^{-1}$ $mouse^{-1}$).

**2.8 Statistical Analysis:** All the results were expressed as mean ± S.E.M. Data of tests were statistically analyzed using one-way ANOVA followed by Turkey's multiple range test, applied for post hoc analysis. The data were considered to be statistically significant if the probability had a value of 0.05 or less.

## 3. RESULTS AND DISCUSSION

In acute study, administration of probiotic LB 405, DNA LB 405, Drug (glyburide,10 mg/kg b.wt) and the combinations as (LB 405 + Drug and DNA LB 405 + Drug) significantly reduced ($p < 0.001$) the blood glucose levels at 2, 4, and 6 h. The onset of hypoglycemic effect of Drug (Group II) was 2 h and that of DNA 405(Group IV) was 4 h (Figure 1). The peak of the effect was attained at 6 h after that it started diminishing and by 24 hours the hypoglycemic effect was no more. The combination of glyburide and DNA 405 (Group VI) exerted an antihyperglycemic effect at 2 h and showed better reduction in glucose levels (37.68 %) than Drug alone (25.13 %).However, LB 405 (Group III) showed 2.71 % decrease in glucose level at 2h in comparison to 16.17 % decrease in DNA LB 405 (Group IV).The antidiabetic activity in various groups in decreasing order is given below:

DNA LB 405 + Drug (Group VI) **>** Drug Control (Group II) **>** DNA LB 405 (Group IV) **>** LB 405 + Drug (Group V) **>** LB 405 (Group III)

Similar to the observations, many studies have proven that probiotics reduces the blood glucose concentrations [20,21,22,23]. Probiotic dahi not only decreases oxidative damage but also increases the antioxidant content and activities of catalase, glutathione peroxidase and superoxide dismutase in diabetic rats [24].Moreover, a marked reduction in pancreatic tissue oxidative damage due to a significant decrease in lipid peroxidation was observed on diabetic rats with probiotic administration[24].

It has been evidenced that when a patient consumes large amount of probiotics, they line the GI tract and feed on this excess glucose, thereby reducing the intestinal absorption of these sugars. With this lowering, the amount of glucose to be converted to glycogen in the liver is also reduced. This mechanism ensures that there is less storage of glycogen, hence less glucose during emergency. Moreover, addition of probiotics in the diet for several weeks has shown to reduce the chances of development of systemic inflammatory induced diabetes by improving the immune response of the body [25].

Sub-acute administration of the LB 405, DNA LB 405, Drug (glyburide,10 mg/kg b.wt) and the combinations as (LB 405 + Drug and DNA LB 405 + Drug) caused a significant ($p < 0.001$) reduction in blood glucose levels as compared to control (Figure 2).Maximum activity was observed by Drug treated group followed by combination of DNA LB 405 + Drug (Group VI) on day 28. However, the combinations (LB 405 + Drug and DNA LB 405 + Drug) resulted in a better and significant ($p <0.001$) response in terms of reduction in blood glucose levels. The

blood glucose level on day 28 shown by the combination group of DNA LB 405 + Drug (Group VI) was 97.6 % whereas that of the Drug (Group V) treated group was 102 %. Moreover, alone DNA LB 405 (Group IV) showed 25.5 % reduction in diabetic level as compared to its use in combination with standard drug i.e. 97.6% reduction (Group VI). The antidiabetic activity in various groups in decreasing order is given below:

*Drug control (Group II) > DNA LB 405 + Drug (Group VI) > LB 405 + Drug (Group V) > DNA LB 405 (Group IV) > LB 405 (Group III)*

Similar to observation with glyburide used in the study, gliclazide an oral antihyperglycemic agent, used for treatment of Non-insulin dependent diabetes mellitus, increases both basal insulin secreation and meal stimulated insulin release. They reported the action of a sulphonylurea with beneficial extrapancreatic effects on glucose level, which may be enhanced by administration of probiotics [4].

Administration of glucose (2.5 g/kg) increased the blood glucose levels significantly ($p < 0.001$) after 30 min of glucose loading in diabetic mice (Figure 3) and normal mice (Figure 4). Probiotic LB405, DNA of LB 405, Drug and combinations (LB 405 and Drug ; DNA LB 405 and Drug) produced significant ($p < 0.001$) increase in glucose threshold within 30 min of glucose loading.

The CpG oligonucleotide is an immunostimulatory sequence present primarily in bacteria [26,27 ,28] and the results of present study using probiotic bacterial DNA might explain one of the mechanisms by which bacterial infections can inhibit the development of diabetes in mice. However, bacterial DNA contains immunostimulatory motifs consisting of a central unmethylated CpG dinucleotide flanked by two 5′ purines and two 3′ pyrimidines [29].These motif stimulates Th1 responses *in vivo*[30].Furthermore, it was showed that in the case of IL-18, protection was associated with systemic activation of Th1-type immunity together with a shift to a Th2 phenotype of the cells infiltrating the islets. Therefore, nonspecific stimulation of the immune system, even by Th1 inducers, is able to reset the ongoing immune response to islet Antigens and arrest the diabetogenic process [31].

The effect of the CpG oligonucleotide on Non Obese Diabetic spleen cells was analyzed *in vitro* , it clearly induced IFN-γ and IL-10 in a dose-dependent manner and it was evident that the effect of the CpG motif favored the release of IL-10. Perhaps the prominence of IL-10 is important in modulating the diabetogenic process [32]. In addition to this, it was observed that the nonobese diabetic (NOD) mouse spontaneously develops insulin-dependent diabetes mellitus (IDDM) as a consequence of an autoimmune process that leads to destruction of the insulin-producing β-cells of the pancreas [33]. It has been shown that the onset of diabetes is preceded by an increase in T cell reactivity toward HSP60 [34]. The CpG oligonucleotide motif present in the bacterial DNA could by itself be used to inhibit the development of diabetes. Therefore, DNA vaccination, either with a vector encoding human HSP60 (pHSP60) or with an empty vector (pcDNA3), diminished the incidence of spontaneous diabetes in females. This effect was accompanied by a significant increase in the number of pancreatic islets remaining free of insulitis [32].

Similar to the present study in which DNA was given intra muscular, earlier studies show that after i.m. injection of a naked expression vector, plasmid DNA is taken up by muscle cells and maintained episomally, allowing the expression of the encoded Antigen [35].Thus, after single or repeated injections of DNA, cellular and/or humoral immune responses to the encoded protein are mounted, and long-lived memory lymphocytes are induced [36] .These memory cells may have regulatory functions and, therefore, might serve as tools for the modulation of autoimmune conditions.

DNA vaccination is also an efficient approach to induce protection against infectious pathogens [37] and cancer [38] and to modulate autoimmune processes [39].Other explanations are possible and more work must be performed to clarify the CpG effect. Nevertheless, the present results encourage the study of therapies aimed to activate pre-existing regulatory networks for the management of diabetes.

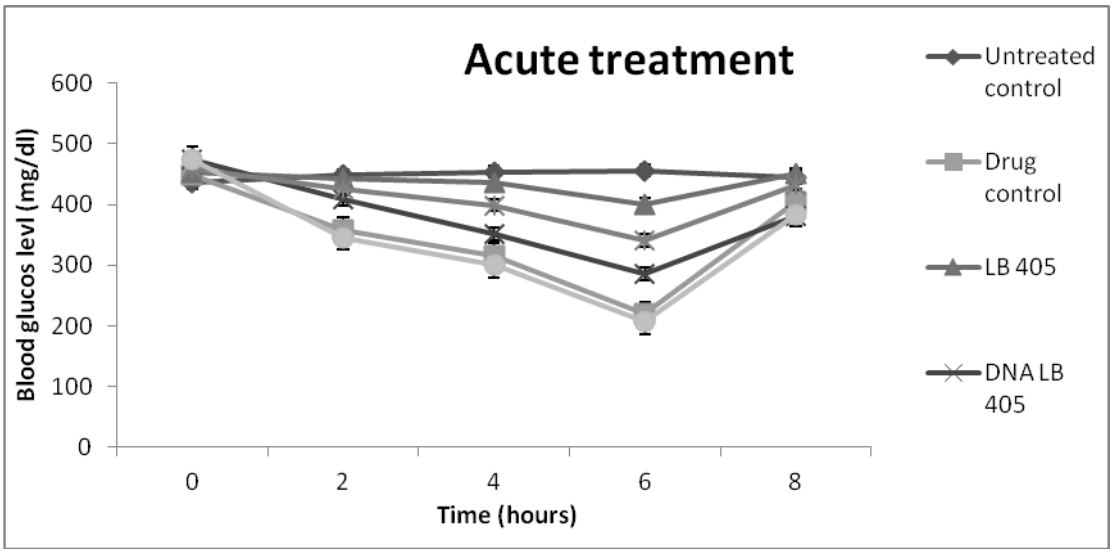


**Fig.1:** Effect of acute treatment of probiotic (LB405), DNA of LB 405, Drug control and combinations (LB 405 and Drug; DNA LB 405 and Drug) on blood glucose level in alloxan-induced diabetes in mice. Blood glucose levels were assessed at regular interval of 0, 2, 4, 6 and 24 h. The results are presented as mean ± SEM (n = 6).

[a] p<0.001 compared with untreated control group.

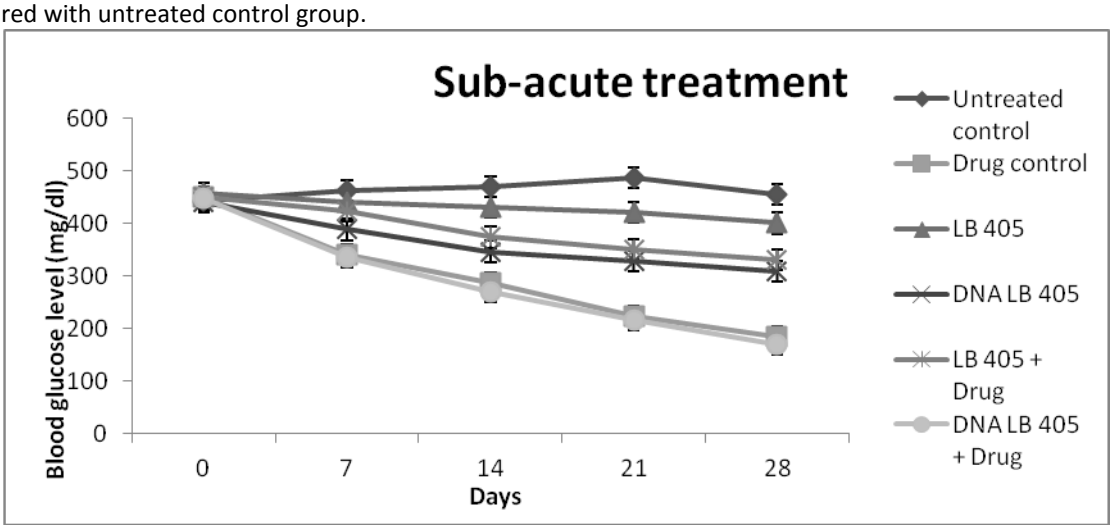


**Fig. 2:** Effect of sub-acute treatment of probiotic (LB405), DNA of LB 405, Drug control and combinations (LB 405 and Drug ; DNA LB 405 and Drug) on blood glucose level in alloxan-induced diabetes in mice. Blood glucose levels were assessed on day 0, 7, 14, 21 and 28. The results are presented as mean ± SEM (n = 6).

[a] p <0.001 compared with untreated control group.

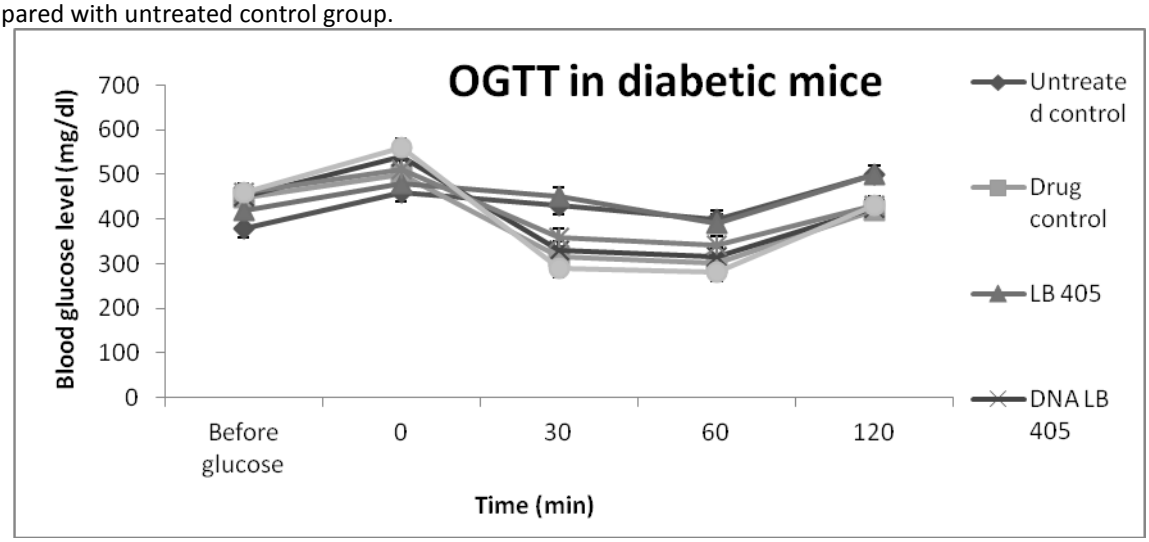


**Fig. 3:** Effect of treatment of probiotic (LB405), DNA of LB 405, Drug control and combinations (LB 405 and Drug; DNA LB 405 and Drug) on blood glucose level in OGTT in diabetic mice. Blood was collected and assessed for blood glucose levels at 0, 30, 60, and 120 min after loading of glucose in diabetic mice. The results are presented as mean ± SEM (n = 6).

[a] p <0.001 compared with untreated control group.

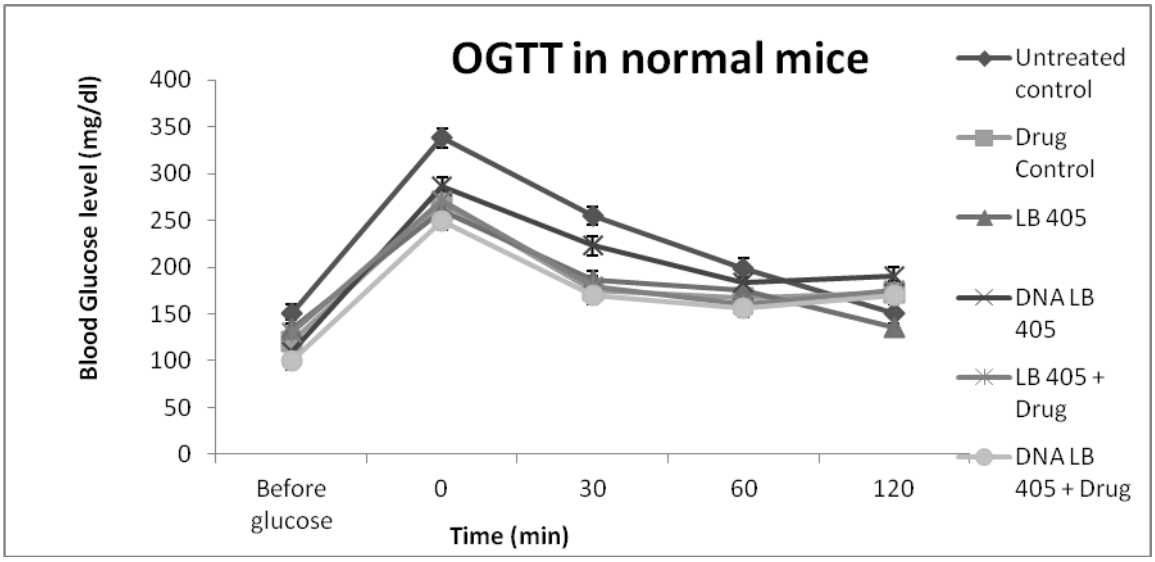


**Fig. 4:** Effect of probiotic (LB405), DNA of LB 405, Drug control and combinations (LB 405 and Drug; DNA LB 405 and Drug) on blood glucose level in OGTT in normal mice. Blood was collected and assessed for blood glucose levels at 0, 30, 60, and 120 min after loading of glucose in normal mice. The results are presented as mean ± SEM (n = 6).
[a] $p<0.001$ compared with untreated control group.

## 4. CONCLUSION

It is concluded that genomic DNA of probiotics should be exploited as a potent antihyperglycemic biotherapeutic agent.

## 5. ACKNOWLEDGEMENT

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