Oral Administration of *Lactobacillus casei* and *Bifidobacterium bifidum* Improves Glucagon Like Peptide-1 and Glucose-Dependent Insulinotropic Polypeptide Level In Streptozotocin Induced Diabetic Rats

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Abstract
The gut microbiome plays significant role in the function and integrity of the gastrointestinal tract. They also maintain immune homeostasis and host energy metabolism. The metabolic products of these intestinal microbes can alter carbohydrate metabolism, nutrient absorption and reduce appetite to promote healthy lifestyle. Intestinal disbiosis observed in metabolic disorders like obesity and diabetes. Restoration of dysbiosed gut microbiome through oral administration of probiotics that may have profound health effect in diabetes. In case of diabetes, reports postulated impaired level of incretin, therefore we explored the effect of oral administration of probiotic bacteria *Lactobacillus casei* NCDC 017 (LC017) and *Bifidobacterium bifidum* NCDC 231 (BB231) alone and in combination on secretion of incretin hormones such as glucagon like peptide-1 and glucose dependent insulinotropic polypeptide. Thirty six male Wistar rats were randomly divided into six groups and diabetes was induced by single dose of streptozotocin (50 mg/kg body weight) in experimental rats intraperitonially except a group of healthy rats. The diabetic rats were daily administered orally with single dose (~10⁷ cfu/ml) of LC017 and BB231 alone and in combination for 28 days. Also, one group of diabetic rats was treated with an anti-diabetic drug, acarbose (10mg/kg body weight) and used a standard control. The change in body weight, sucrose tolerance test, GLP-1, GIP level in serum and GLP-1 level in different part of intestine were observed. The results have shown

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Introduction

Glucagon like peptide-1 (GLP-1) and glucose dependent insulinotropic polypeptide (GIP) are incretin hormones released from intestinal enteroendocrine L and K cells respectively in response to food intake, especially by carbohydrates and fat rich diet. Increment in insulin hormone production and pancreatic beta cell proliferation. GLP-1 play important role in stimulation of glucose dependent insulin secretion, augmentation of beta-cell mass, inhibition of glucagon release, decrease gastric emptying and food intake. The physiological action of GLP-1 and GIP are very much similar and additive. Like GLP-1, GIP also have insulinotropic effect, GIP act through by binding to specific GIP receptor present on pancreatic beta cells and enhance exocytosis of insulin containing granules. GLP-1 and GIP decreases apoptosis and increase proliferation rate of pancreatic beta cells. Wang et al. reported that GIP induces expression of proinsulin gene and enhance the secretion of insulin hormone. GLP-1 and GIP incretin hormones have very short half life because these are rapidly degraded by dipeptidylpeptide-4 (DPP-4) enzyme. DPP-4 is located in endothelial and epithelial cells of intestine. Several studies have reported an impaired incretin effect in diabetes patients that attributed defective insulin secretion. Incretin based therapy are most recently approved class of therapeautic agents for treatment of diabetes, especially via DPP-IV inhibitors and GLP-1 receptor agonist. Sitagliptin and saxagliptin are DPP-IV inhibitors which exert their action through prevention of incretin degradation. Exendin-4 and liraglutide are GLP-1 receptor agonist having structurally resemblance to GLP-1. Exendin-4 was approved for treatment of diabetes in the U.S. and liraglutide in the Europe in 2005 and 2006, respectively. These drugs are chemically synthesized and not safe for regular and long-term use, having some adverse effects like weight gain, hypoglycaemia, fever, gastrointestinal discomfort and high cost of drugs. The side effects associated with incretin based drugs necessitated for safer alternatives, including enhancing endogenous production of incretin hormones in diabetic subjects. Many studies supported that probiotic strains have high potential in treatment of diabetes and obesity. It has been shown that probiotic containing foods delay the streptozotacin induced diabetes and protect islets of pancreatic beta-cells from damage, delaying the onset of T2DM and prevent complications associated with DM. Balakumar et al. reported that probiotics treatment had ability to normalize the level of GLP-1 which is impaired in high fat diet induced obesity. Probiotics are live microorganism which, when administered in adequate amount confer a health benefit to the host. Probiotics are used as potential modulators of the intestinal microbial flora in a valuable manner. Probiotics have antidiabetic, antimutagenic, anti-inflammatory, antioxidants, immunomodulatory and antiobesity activities and provide a number of health benefits to host through modulation of the gut microbiota. Yadav et al. reported that administration of various combinations of probiotics to obese mice protected from body weight gain, reduced food intake and insulin resistance. Various metabolic disorders like diabetes and obesity create imbalance of gut microbiota. It is reported that proportion of Firmicutes and Clostridia were higher in the diabetic patients compared to healthy individuals, patients in the pre-diabetes and T2DM groups had significantly increased level of Betaproteobacteria compared with healthy group so by administration of adequate amount of probiotic, use to recover intestinal microbiota with beneficial bacteria. Species of Lactobacillus and Bifidobacterium are most commonly used microbes as probiotics. Zeng et al. reported that strains of Lactobacillus and Bifidobacterium have DPP-4 inhibitory activity. Thus, these probiotics have potential application in
management of diabetes. Our previous study has shown potential hypoglycaemic and antioxidant activity of *L. casei* and *B. bifidium* in diabetic animal models. Therefore, the present study was designed to investigate the effect of probiotic treatment on incretin hormone secretion in experimental rat models.

**Materials and Methods**

**Materials**

Streptozotocin and acarbose were procured from Sigma Aldrich (St. Louis, Missouri, USA). Blood glucose estimation by Accu check one touch glucometer (Johnson & Johnson, Mumbai, India). De Man Rogosa and Sharpe (MRS) agar and broth media were purchased from HiMedia Laboratories (Mumbai, India). Sitagliptin purchased from Merck Serono Co., Ltd, Guanghoou, china. GLP-1 and GIP were estimated by using standard ELISA kit purchased from RayBio®. BioAssay Systems.

**Bacterial strains**

LC017 and BB231 probiotic strains those were used in this study, procured from National collection of dairy culture, National Dairy Research Institute, Karnal, India.

**Animal Handling**

Male Wistar rats, weighted about 170-210 gm were procured from the animal research division, Defence Research & Development Establishment, Gwalior, India. This study has been approved by the Institutional Animal Ethical committee (IAEC) and application to be submitted to the committee for the purpose of control and supervision of experiments on animals (CPCSEA), New Delhi (Reg.No. BU/PARMA/IAEC/a/16/12). All rats were housed in animal room in plastic cages with husk bedding and cages were cleaned and changed daily. Animal room maintained with 12-h light-dark cycle at 22±2 °C temperature and humidity 55±5%. All rats were provided normal pellet diet and RO water *ad libitum*. The rats were acclimatised for one week before starting experiments.

**Induction of Diabetes**

Diabetes was induced in rats by single dose of freshly prepared streptozotocin solution (50 mg/kg body weight) in sterile 0.1 M citrate buffer (pH 4.5) to overnight fasted animal intraperitoneally. Diabetes was verified after 96 hour by evaluating blood glucose level. FBG and PBG were taken regularly till stable hyperglycaemia condition achieve. Rats having blood glucose level 250mg/dl or more were used in this study.

**Preparation and Dosing of LC017 and BB231**

Lyophilised bacterial strains were revived in MRS broth medium and incubated at 37°C in anaerobically in an anaerobic system Mark II (Anaero Gas Pack, LE002. HiMedia, India) for 48 h. After incubation period, 1 mL of inoculum was diluted six times by serial dilution method in sterile distilled water. 100 uL suspension of last successive serial dilution was inoculated on MRS agar plate and after incubation 50-60 colonies appear in each plate. This plate was used to prepare dose for animals. The concentration of last dilution was 56×10^7 cfu/mL. One colony picked using sterile loop and dissolved in 1 mL sterile water and mixed with sterile micropipette. All the procedure of preparation of bacterial dose took place in laminar air flow. Same method was used to prepare both bacterial strains. Freshly prepared single daily dose of LC017, BB231 alone and combination administered to treated groups of animals for 28 days.

**Experimental Design**

The animals were divided into six experimental groups each contain 6 rats (n=6).

| Group   | Treatment                                | Dose                                    |
|---------|------------------------------------------|-----------------------------------------|
| Group-1 | Healthy control                          | Normal rat + single daily dose of sterile distil. water 1mL |
| Group-2 | Untreated diabetic                       | Single dose of streptozotocin 50mg/kg b.w. + single daily dose of sterile water 1mL |
| Group-3 | Treated group                            | Single daily dose of LC017 (~10^7cfu/mL) |
| Group-4 | Treated group                            | Single daily dose of BB231 (~10^7cfu/mL) |
| Group-5 | Treated group                            | Single daily dose of LC017 and BB231 (~10^7cfu/mL) |
| Group-6 | Standard group                           | Single daily dose of acarbose (10 mg/kg b.w.) |

The drugs and bacterial doses were administered orally by using feeding needle once daily for 28 days, continuously.
**Body Weight Measurement**
The body weight was measured of each rat before treatment and every week during the experiment.

**Sucrose Tolerance Test**
For carbohydrate tolerance test, the rats were fasted overnight and administered sucrose (2 g/kg b.w.) orally dissolved in 1 mL of distilled water in combination with treatment. Blood was sampled from tail vein at 0, 30, 60, 90, and 120 min after sucrose administration to measure blood glucose level. Blood glucose level was determined by using Accu. check One touch glucometer.

**Serum GLP-1 and GIP Estimations**
At the end of the experiment (i.e. on 28th day), rats were given glucose load (2 g/kg b.w.) after overnight fasting by gavage. Blood was sampled at 0, 20, 30, 40, 50 and 60 min from tail vein and collected into chilled tubes containing EDTA and DPP-4 inhibitor (sitagliptin) for serum GLP-1 and GIP analysis. Quantification of GIP and GLP-1 was accomplished by rat ELISA kit (RayBio® rat GLP-1 enzyme immunoassay kit, EIA-GLP-1 and RayBio® rat GIP enzyme immunoassay kit, EIA-GIP) using Thermo Scientific Varioskan Flash Spectral Scanning Multimode Reader. After collection of blood from tail vein, rats were sacrificed under mild ether anaesthesia. Total loss of consciousness was confirmed by toe-pinch. Blood sample was collected by heart puncture and serum was isolated by centrifugation of blood sample at 3000 rpm for 5 min at 4°C and stored in -80°C until analysis.

**Extraction and Estimation of Intestinal GLP-1**
Intestinal segments (jejunum, ileum, colon and cecum) were immediately isolated from sacrificed animals and washed with phosphate buffer-saline. Different segments of intestine were homogenised separately using ethanol/acid (5:1 v/v) solution (5 mL/g tissue) at 4°C. Homogenised tissue kept for 24 h at 4°C and after that centrifuged at 10,000 g for 20 min, at 4°C. The supernatant was transferred and neutralized with 1 mol/l of NaOH and used for intestinal GLP-1 estimations by using ELISA kits (RayBio® rat GLP-1 enzyme immunoassay kit, EIA-GLP-1) according to manufacturer’s instruction.

**Table 1: Effect of Probiotic treatment on change in body weight (g) of normal and diabetic rats**

| Groups               | Treatment          | Day 1  | Day 7  | Day 14 | Day 21 | Day 28 |
|----------------------|--------------------|--------|--------|--------|--------|--------|
| Normal control       |                    | 231.2±1.32 | 235.6±1.57 | 249.01±1.74 | 260.2±2.28 | 272.7±1.94 |
| Diabetic control     |                    | 229.82±2.3 | 208.61±2.85*** | 199.4±4.01*** | 185.11±5.19*** | 168.63±6.24*** |
| L. casei treated     |                    | 226.45±2.56 | 209.7±2.90*** | 197.16±1.93*** | 192.5±2.07*** | 187.38±1.48*** |
| B. bifidum treated  |                    | 225.7±2.2 | 210.7±1.35*** | 202.78±1.77*** | 194.05±2.09*** | 187.27±2.46*** |
| L. casei and B. bifidum treated |        | 224.93±1.8 | 206.5±2.13*** | 212.4±2.12*** | 221.8±1.59*** | 230.11±1.20*** |
| Combination treated  |                    | 228.78±2.13 | 209.6±2.54*** | 212.8±2.56*** | 223.1±2.96*** | 232.3±3.56*** |
| Acarbose             |                    | 225.7±2.2 | 210.7±1.35*** | 202.78±1.77*** | 194.05±2.09*** | 187.27±2.46*** |

The Values are Mean ± SEM (n = 6 animals/group). * = P < 0.05, ** = P < 0.01, *** = P < 0.001 compared to positive (healthy) control. a = P>0.05, b = P < 0.05, c = P < 0.01, d = P < 0.001, compared to negative (diabetic) control.

**Statistical Analysis**
All the data were presented as mean ± standard error of mean (SEM). The data analyzed by statistical software (statistical package for social sciences, SPSS Version 20.0, IBM) using one-way ANOVA.
followed by Tukey’s multiple range post hoc tests. The values were considered significantly different at $P<0.05$.

**Result**

**Effect of Treatment on Body Weight**

During 28 days of experiment, diabetic rats were found to have significant ($p < 0.001$) weight loss, compared to healthy control group. The body weight was significantly ($p < 0.001$) increased for the acarbose and combination treated groups compared to diabetic group (Table 1). Results indicate that treatment with probiotics affect body weight and could effectively improve weight loss in diabetic animal.

**Effect of Treatment on GLP-1 Level in Serum**

Significant ($P < 0.01$) decrease in GLP-1 level was observed in non treated diabetic rats as compared to healthy rats. Significant increase ($p < 0.01$) in GLP-1 level after 28 days of treatment with LC017, BB231 and combination in therapeutic models (Fig. 1). Diabetic animal treated with standard drug showed significantly increased and normalized GLP-1 level compared to healthy control rats.

![GLP-1 level in serum at the end of experiment](image)

Fig. 1: GLP-1 level in serum at the end of experiment. Result were presented as mean $\pm$ SEM ($n=6$ animal/group). $a= P>0.05$, $b= P < 0.05$, $c= P < 0.01$, $d=P<0.001$ compared to normal control. $*= P < 0.05$, $**= P < 0.01$, $***= P < 0.001$ compared to diabetic control

**Effect of Treatment on GIP Level in Serum**

Similar to GLP-1, diabetic rats showed a significantly ($p< 0.01$) decreased level of GIP as compared to nondiabetic healthy rats. Administration of LC017, BB231 and combination therapy significantly ($< 0.01$) increased the level of GIP in treated rats as compared to diabetic control (Fig. 2).
Fig. 2: GIP level in serum at the end of experiment. Result were presented as mean ±SEM (n=6 animal/group). a= P>0.05, b= P < 0.05, c= P < 0.01, d= P<0.001 compared to normal control. 
*= P < 0.05, **= P < 0.01, ***= P<0.001 compared to diabetic control.

Fig. 3: GLP-1 Content in different part of the Intestinal tract. Result were presented as mean ±SEM (n=6 animal/group). a= P>0.05, b= P < 0.05, c= P < 0.01, d= P<0.001 compared to normal control. 
*= P < 0.05, **= P < 0.01 , ***= P<0.001 compared to diabetic control.
Effect of Treatment on GLP-1 Level In Intestine
Among different parts of intestine, GLP-1 content was highest in colon region. GLP-1 level was significant decreased (p<0.01) in all animals among different parts of intestine in diabetic rats. After 28 days of treatment, GLP-1 level significantly increased in diabetic rats (Fig. 3).

Effect of Treatment on Sucrose Tolerance
After 28 days of experiment, sucrose tolerance test was performed and we found that maximum blood glucose level reach at 60 min. Blood glucose level of group treated with combination of both LC017 and BB231 were slightly higher than group treated with standard antidiabetic drug acarbose. Oral administration of LC017 and BB231 treatment reduced postprandial blood glucose after sucrose challenge like acarbose. Glucose level significantly (p<0.01) higher in untreated diabetic rat compared to rats treated with probiotics. (Fig.4). Rats treated with combination of both bacteria showed significantly deceased level of glucose after sucrose tolerance test.

Discussion
Probiotics are the widely used nutritional supplements and therapeutic modality in the management of diabetes and obesity. Probiotic bacteria are defined as living microorganism which confers health benefits to their host when administered in adequate amounts. Various studies reported the potential health benefits of Bifidobacterium and lactic acid bacteria such as antitumor activity, antimicrobial activity, improvement of immune system and gastrointestinal microbiota. Manaer et al. reported significant hypoglycaemic effect in diabetic rats through enhancing the release of GLP-1 and improving pancreatic beta cells, by using camel milk fortified with various strains of LAB (Lactic acid bacteria) and yeast. Our previous study reported...
that treatment with *L. casei* and *B. bifidum* alone and combination (single daily dose 1×10^7 cfu/mL) ameliorates antioxidant stress and hyperglycemia. So, in the present study we have explored the mechanism by which the probiotic treatment promote hypoglycaemic activity, emphasizing on the effect of probiotic treatment on incretin level. We hypothesized that oral administration of *L. casei* and *B. bifidum* alone and in combination might result in change in the level of incretin hormones. To delineate the process we measured the level of incretin hormones especially GLP-1 in serum and intestine that regulate food intake. After 28 days of experiment, we found that hunger reducing hormone GLP-1 was significant decrease in streptozotocin induced diabetic groups as compared to control groups however, oral administration of LC017 and BB231 treatments increase the level of GLP-1 in therapeutic models but interestingly therapeutic group treated with combination of both *L. casei* and *B. bifidum* showed significantly increase and normalized GLP-1 level similar to acarbose treated group. These results illustrate that increased the level of GLP-1 correlate with beneficial metabolic effect of LC017 and BB231. Several studies supported that probiotic treatment enhance the secretion of incretin hormone have been correlated to increase the level of insulin hormone by the recovery of pancreatic islets due to antiapoptotic, regeneration activity of GLP-1 and the secretion of GLP-1 associated with modulation of intestinal microbiota. In the present study, body weight was significantly reduced in diabetic group, this sudden reduction in weight due to degeneration of lipid found in tissue and muscles as lipid involved in gluconeogenesis, in hyperglycaemic condition hence muscles and lipid tissues are important contributor of weight gain. However, body weight of rats was significantly increased after 28 days treatment with combination of both probiotic strains. The possible mechanism of treatment is associated with increase the level of insulin that in turn improves glycemic control and prevents body weight loss. The observation is supporting previous results. 

**Conclusion**

The result of present study showed that administration of LC017 and BB231 have significant hypoglycaemic potential by increasing the levels of incretin hormones. These incretin hormones improved physiology of islets of pancreatic beta cells and increased the secretion of insulin hormone. The study further supported that combined treatment of *L. casei* and *B. bifidum* possesses higher potential as compared to treatment with each one alone.

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**Conflicts of Interest**

The authors declare no conflict of interest.

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