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RESEARCH PAPER

Oral Delivery of Glucagon Like Peptide-1 by a Recombinant *Lactococcus lactis*

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ABSTRACT

Purpose To develop a live oral delivery system of Glucagon like peptide-1 (GLP-1), for the treatment of Type-2 Diabetes.

Methods LL-pUBGLP-1, a recombinant *Lactococcus lactis* (*L. lactis*)) transformed with a plasmid vector encoding GLP-1 cDNA was constructed and was used as a delivery system. Secretion of rGLP-1 from LL-pUBGLP-1 was characterized by ELISA. The bioactivity of the rGLP-1 was examined for its insulinotropic activity on HIT-T15 cells. Transport of rGLP-1 across MDCK cell monolayer when delivered by LL-pUBGLP-1 was studied. The therapeutic effect of LL-pUBGLP-1 after oral administration was investigated in ZDF rats.

Results DNA sequencing and ELISA confirmed the successful construction of the LL-pUBGLP-1 and secretion of the active form of rGLP-1. *In vitro* insulinotropic studies demonstrated that LL-pUBGLP-1 could significantly ($p < 0.05$) stimulate HIT-T15 cells to secrete insulin as compared to the controls. When delivered by LL-pUBGLP-1, the GLP-1 transport rate across the MDCK cell monolayer was increased by eight times ($p < 0.01$) as compared to the free solution form. Oral administration of LL-pUBGLP-1 in ZDF rats resulted in a significant decrease (10–20%, $p < 0.05$) in blood glucose levels during 2–11 h post dosing and a significant increase in insulin $AUC_{0\text{-}11h}$ (2.5 times, $p < 0.01$) as compared to the free solution.

Conclusion The present study demonstrates that *L. lactis* when genetically modified with a recombinant plasmid can be used for the oral delivery of GLP-1.

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KEY WORDS GLP-1 · *L. lactis* · normal flora · oral protein delivery · type-2 diabetes

ABBREVIATIONS

GLP-1	Glucagon like peptide-1
L. lactis	*Lactococcus lactis sub sp. lactis*
LL-pUB1000	*Lactococcus lactis* transformed with pUB1000 plasmid
LL-pUBGLP-1	*Lactococcus lactis* transformed with pUBGLP-1 plasmid
M17G	M17 growth media supplemented with 5% glucose
pUB1000	Expression host plasmid vector
pUBGLP-1	Recombinant plasmid with GLP-1 c-DNA
rGLP-1	recombinant GLP-1 secreted extracellularly by LL-pUBGLP-1
T2DM	Type 2 Diabetes mellitus

INTRODUCTION

Glucagon like peptide −1 (GLP-1) is an incretin that has emerged as a promising therapeutic agent for the treatment of T2DM. Incretins are the hormones that are produced by gastrointestinal tract in response to nutrient entry and are necessary for the maintenance of glucose homeostasis (1,2). Deficiency in incretin function usually results in the pathogenesis of diabetes mellitus (3). GLP-1 is a 30 amino acid peptide secreted by intestinal L-cells in response to meal ingestion with lipids and carbohydrates (4). GLP-1 not only enhances the insulin secretion but also suppresses the secretion of glucagon in a glucose-dependent fashion. The insulinotropic effect of GLP-1 is strictly glucose dependent (5). GLP-1 exerts its effect by acting on G-protein-coupled receptors located on the pancreatic β-cells (6). Furthermore, GLP-1 also exerts its action by promoting proliferation and neogenesis of

pancreatic β-cells. GLP-1 based therapy; a contemporary class of drugs for T2DM is therefore, attracting considerable attention on account of its immense potential to serve as a safe and unique anti-diabetic drug.

In spite of these above mentioned important physiological effects of GLP-1 on type-2 diabetes, the native form of GLP-1exerts extremely short half-life *in vivo* (~2–5 min). This short half-life of GLP-1 is due to its rapid inactivation by a ubiquitously present enzyme system, dipeptidyl peptidase 4 (DPP-IV), which significantly hampers its clinical use.

To overcome this challenge, currently, two new classes of drug products that target incretins are available clinically: GLP-1 receptor agonists or mimetics (GLP-1 analogues that are resistant to DPP-IV) and DPP-IV inhibitors. Januvia® (Sitagliptin), and Onglyza® (Saxagliptin) are examples of potent inhibitors of DPP-IV enzyme that result in longer circulating half-life of GLP-1. The major advantage of DPP-IV inhibitors is that they are available as oral dosage form. But a point to consider about DPP-IV inhibitor is that since many other hormones, neuropeptides, and chemokines are substrates of DPP-IV enzyme, chronic use of DPP-IV inhibitors may negatively impact other crucial biochemical pathways in the body that are governed by this enzyme system (7,8).

Also, acute pancreatitis along with severe hemorrhagic pancreatitis is emerging as a major concern in patients who are taking DPP-IV inhibitors alone or as a combination therapy (9–11). Byetta® (exenatide), manufactured by Amylin Pharmaceuticals is among the first GLP-1 receptor agonists/mimetic class approved by FDA in 2005. Byetta is available as twice daily injection with improved biological half-life of 2.4 h (12). Victoza® (Liraglutide) another GLP-1 receptor agonist with an extended duration of action of 13 h requires it to be injected once daily and most recently Bydureon® (exenatide extended release), manufactured by Amylin Pharmaceuticals is available as once weekly injection. Although successful formulations of GLP-1 receptor agonists with extended duration of action are now available, it is still administered by injection.

Therefore, there is an imperative need for oral formulation of GLP-1. Although oral administration of protein drugs poses serious challenges, it still remains the most preferred route because of its high levels of patient acceptance and long term compliance, which increases the therapeutic value of the drug. Some studies have shown that adequate absorption of protein can be achieved after oral administration, if there is enough protein drugs present on the intestinal epithelium (13,14). Therefore, we have hypothesized that a system that can synthesize and secrete GLP-1 right on the intestinal epithelium and render a high amount of the protein on the absorption surface by minimizing its exposure to the unfavorable intestinal environment can be used as a potential novel oral delivery system for GLP-1. We have attempted to develop such a device using recombinant *Lactococcus lactis subsp. lactis* (*L. lactis*) as the delivery system.

L. lactis is considered as a normal flora of human body that belongs to a group of bacteria called Lactic acid bacteria (LAB) (15–17). Normal flora is composed of non-pathogenic bacteria that exist in several areas of human body such as intestine, nostril and vagina (18). *L. lactis*, one of the safest strains of LAB is emerging as a potential candidate for the delivery of many heterologous proteins (19–21). Their traditional use in the food industry has confirmed their safety profile and hence holds generally regarded as safe status (GRAS) by FDA.

Several studies demonstrated the usefulness of LAB as probiotics for human and animals (22–24). Lactobacillus strains have been used as an attractive candidate for expressing foreign antigens and mucosal immunization (25–30). Genetically modified *L. lactis* has been also shown to secrete interleukin-10 that can be utilized in the treatment for inflammatory bowel disease (31,32). Recombinant *L. lactis* has also been used as an expression system for mucosal delivery of murine IL-2 and IL-6 (33–35) and for secretion of interleukin-12 (36).

Studies have also demonstrated that probiotic lactobacilli, following oral delivery, can survive in the human GI tract. In a study where human feeding of *L. lactis* was performed, a substantial portion of *L. lactis* was seen to survive the passage of the gastrointestinal tract within 3 days after consumption (37). Previous work from our laboratory has demonstrated that genetically modified *L. lactis* encoding the gene for beta-lactamase (a 22 kD protein) enhanced the oral bioavailability of beta-lactamase in rats (38).

Therefore, designing a recombinant *L. lactis* containing GLP-1 gene that can secrete biologically active form of GLP-1 extra-cellularly can be explored as a potential vehicle for GLP-1 delivery. Based on the findings of above mentioned literatures, in this study, we constructed a recombinant *L. lactis* (LL-pUBGLP-1) as a novel oral delivery system for native form of GLP-1. The study aimed at the secretion of biologically active form of GLP-1 from LL-pUBGLP-1. The study also investigated the *in vitro* transport of secreted GLP-1 across MDCK cell monolayer. Finally, the efficacy of LL-pUBGLP-1 after oral administration for treatment of T2DM was tested in Zucker Diabetic Fatty rats (ZDF).

MATERIALS AND METHODS

Reagents

Madin-Darby canine kidney cell line (MDCK), Syrian Hamster Pancreatic beta cells (HIT-T15), and untransformed *Lactococcus lactis* subsp. *lactis* (*L. lactis*) were purchased from American Type Tissue Culture (ATCC) (Rockville, MD,

USA). Bacto M17 broth and Bacto agar were purchased from Becton Dickinson (Sparks, MD, USA). Dulbecco's phosphate buffer saline solution, pH 7.4 (DPBS), Dulbecco's modified eagle medium (DMEM), Ham's F12K medium, Hank's balanced salt solution (HBSS), 0.25% trypsin with 0.2 g/l ethylenediamine tetra acetic acid (EDTA), Fetal bovine serum (FBS), dialyzed horse serum, sodium pyruvate (11 mg/ml) and non-essential amino acids (100 X) were obtained from Hyclone (Logan, UT, USA). GLP-1 ELISA kits (EGLP-35 K) were purchased from Linco Research (St. Louis, MO, USA) and Ultra sensitive Rat Insulin ELISA kits from Mercodia. Tissue culture treated transwell® inserts, (0.4 μm pore size, 4.7 cm^2 surface area), and culture flasks were purchased from Costar Corporation (Cambridge, MA, USA). GLP-1, erythromycin, d-mannitol-[1-^{3}H(N)], d-l-propranolol-[4-^{3}H] hydrochloride and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Construction of LL-pUBGLP-1

The plasmid pUB1000 kindly donated by Dr. H. F. Jenkinson (39) was used as an expression host vector. This vector carries a lactococcal promoter region P1 and a signal peptide coding sequence usp45 followed by *Sal*I and *Bam*HI restriction site (Fig. 1a). The GLP-1 cDNA sequence was obtained from GenBank (NM_002054). The GLP-1 cDNA fused with *Sal*I and *Bam*HI cDNA restriction sites (113 bp) with overhangs was synthesized from a commercial source (EzBiolabs, Carmel, IN, USA). Figure 1b shows a schematic representation of the GLP-1 cDNA. The pUBGLP-1 was constructed by inserting GLP-1 cDNA into the *Sal*I/*Bam*HI cloning site of pUB1000 vector. The constructed plasmids were confirmed by DNA sequencing and PCR. The pUBGLP-1 plasmids were transformed into competent *L. lactis* cells by electroporation (40). The cell suspension was spread on the agar plates containing erythromycin and incubated at 37°C. After two days, the colonies were selected and cultured in M17 broth at 37°C. Aliquots of the culture were collected at regular intervals and subjected to cell counting by serial dilutions, the measurement of absorbance at 600 nm and GLP-1 analysis by ELISA as described by the manufacturer's protocol.

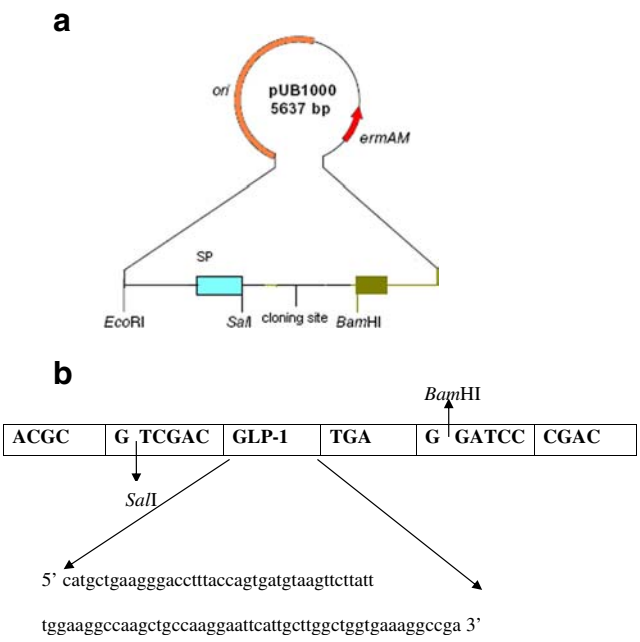

Fig. 1 (**a**) Plasmid map of pUB1000. Adapted from (39). (**b**) GLP-1 c-DNA (coding sequence in GLP-1 c-DNA is shown with *big arrows*) along with *Sal*I and *Bam*HI restriction sites. The site of cleavage by *Sal*I and *Bam*HI is indicated by *small arrows*.

Cell Culture of HIT-T15 Cells

HIT-T15 cells were routinely maintained in Ham's F12K medium supplemented with 2 mM L-glutamine 1.5 g/l sodium bicarbonate, 10% (v/v) dialyzed horse serum, 2.5% (v/v) fetal bovine serum, 100 I.U./ml penicillin, 100 μg/ml streptomycin, sodium pyruvate (0.11 mg/ml) and 1% (v/v) non-essential amino acids. The cells were grown in 75 cm^2 flasks and incubated at 37°C in a humidified incubator with 5% CO_2. Upon 75% confluence, the cells were harvested by 0.25% trypsin EDTA and seeded in a 6-well plate at a density of 5×10^4 cells per well. The cells were then grown for 4–5 days before the insulinotropic studies.

Insulinotropic Effect on HIT-T15 Cells

The HIT-T15 cells were grown for 4–5 days in 6-well plates until confluent. Then the cells were washed twice with glucose-free HBSS containing 0.1% bovine serum albumin with 30-min incubation each time. The medium was then replaced with 1 ml logarithmic phase culture of LL-pUBGLP-1 suspension (~10^{10} cfu/ml) in M17 medium (absence of glucose) or M17G medium (M17 + 5 mM glucose). The cells were incubated for 1 h. Standard GLP-1 solutions (50 pM) in M17 and M17G medium were used as positive controls. Blank M17, M17G, logarithmic phase culture of LL-pUB1000 (~10^{10} cfu/ml) in M17 and M17G were used as negative controls. At the end of the 1 h incubation, an aliquot (500 μl) of the medium was collected and centrifuged for 5 min at 3000×*g*. The bacterium-free supernatant (50 μl) was subjected for insulin assay by ELISA (Mercodia, NC). Finally, the HIT-T15 cells were lysed with 1 ml of 0.1% triton X-100 in 1.0 N sodium hydroxide solution for total protein analysis by bicinchoninic acid (BCA) method. Insulin release was normalized to the cellular protein content.

MDCK Cell Culture

The MDCK cell line was obtained from ATCC at serial passage number of 55.

The MDCK cells were routinely maintained in supplemented DMEM medium (s-DMEM) composed of 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 10% (v/v) fetal bovine serum, 100 I.U./ml penicillin, 100 μg/ml streptomycin, sodium pyruvate (0.11 mg/ml) and 1% (v/v) non-essential amino acids. The cells were grown in 75 cm^2 flask at 37°C in a 5% CO_2 –95% air with high humidity. Upon 75% confluence, the cells were harvested by 0.25% trypsin EDTA and re-suspended in s-DMEM. The cells were sub-cultured for several times, and the cells used in this study were from passage number 55–67.

MDCK Cell Viability in M17G

To select a common growth media for MDCK and the bacterial cells, the MDCK cells were seeded onto the 96 well plates at a density of 5000 cells/well and further incubated at 37°C in a humidified incubator with 5% CO_2 for 48 h. The growth media were then replaced with either 100 μl of s-DMEM, M17 supplemented with 0.5 (w/v) glucose (M17G) or s-DMEM and supplemented M17G (1:1), and the cells were further incubated for 3 and 6 h under normal cell culture conditions. After the incubation, the cell viability was estimated by the standard protocol of MTT assay.

MDCK Cell Viability in the Presence of LL-pUBGLP-1

After the MDCK cells formed a confluent monolayer in the transwells, the culture media were replaced with DPBS and the cells were incubated for 30 min. This washing process was repeated once. Viability experiments were started by adding 1.5 ml of freshly made LL-pUBGLP-1 (~10^8 cfu/ml) in M17G medium supplemented with 5 μg/ml of erythromycin on to the apical side and 1.5 ml M17G media on to the basal side. The plate was centrifuged at 45×*g* for 5 min (Beckman GPR Centrifuge) and was placed inside the incubation chamber. The MDCK cell viability was then determined by trypan blue assay at 3 h and 6 h after the addition of LL-pUBGLP-1. This study was conducted in total of 6 wells for each treated and control for each time point.

MDCK Monolayer Integrity During the Transport Experiment

To investigate the integrity of MDCK monolayer in the presence of LL-pUBGLP-1, 1.5 ml of LL-pUBGLP-1 (~10^8 cfu) was added onto the apical chamber of the confluent MDCK monolayer in the transwell inserts. The TEER values were then measured from 0 to 6 h at each hour after the addition of LL-pUBGLP-1. TEER was also monitored for the cells treated with s-DMEM that served as a control. For each series of measurements, background resistance was measured using unseeded wells each of which had a value of 80Ω·cm^2. This value was used as a correction factor for each measurement. Each experiment was performed in triplicate to obtain the average and standard deviation.

Integrity studies were also carried out by investigating the transport of ^{3}H-mannitol and ^{3}H-propranolol across the MDCK cell monolayers grown on transwell plates. First, the medium was aspirated and the MDCK cell monolayers were washed twice with DPBS for 30 min at 37°C. To the basolateral chamber, 2.6 ml of M17G was added, and to the apical side, 1.5 ml of the freshly made LL-pUBGLP-1 (~10^8 bacterial cells/well) in M17G containing either 66.7 nM d-mannitol-[1-^{3}H(N)] or 35.5 nM dl-propranolol-[4-^{3}H] hydrochloride was added. Samples (500 μl) were withdrawn from the basolateral side and replaced with equal amount of the fresh media. The samples were withdrawn at 0, 0.5, 1, 2, 3, 4, 5 and 6 h. At the end of the experiment, 500 μl of sample was also taken from the apical side. All the samples were collected in scintillation vials to which 10 ml scintillation cocktail ScintiVerse (Fisher Chemical Co., Pittsburgh, PA) was added. The samples were then vortexed and counted by a Packard Tricarb LSC Counter for 5 min each to obtain the total count per minute (cpm).

Transport of rGLP-1 Through MDCK Monolayer

The MDCK cell line obtained from ATCC at serial passage number of 55 was routinely maintained in the supplemented DMEM medium (s-DMEM). The cells were seeded onto the polycarbonate filter inserts (0.4 μm pore size) of the 6-well Transwell™ plates (Costar, Cambridge MA, USA) at a density of 3x10^4 cells/cm^2 and incubated at 37°C under normal cell culture conditions. The basolateral chamber of the transwell plate was filled with 2.6 ml of s-DMEM. The media in both the apical and basal chambers were replaced every alternate day. The TEER was monitored periodically. The transport studies were carried out after 5–6 days post seeding when the monolayer became confluent. The basal media of each well was replaced with 1.5 ml of fresh M17G. Media in the apical chamber was replaced with either 1.5 ml of freshly made LL-pUBGLP-1 in M17G (~2x10^8cfu/ml) or 1.5 ml of standard GLP-1 solution (70 pM) in M17G.

The plate was centrifuged at 45×*g* for 5 min and incubated at normal cell culture conditions. At the predetermined time periods, 200 and 500 μl samples from the apical and the basal sides respectively were withdrawn and replaced with equal volume of the fresh M17G. The samples were subjected for GLP-1 assay by ELISA as described by the manufacturer's protocol.

Animal Studies

The animal research protocol was approved by the Biosafety and Institutional Animal Care and Use Committee of the St. John's University and conformed to the NIH guide for the use

and care of laboratory animals. Male ZDF rats, 10 weeks of age, 420±50 g purchased from Charles River Laboratories, Wilmington, MA, were used for the following experiments. All the rats were 12–14 weeks of age when used for the study and were at their fully developed T2DM stage.

Pharmacodynamic Effects of LL-pUBGLP-1 in ZDF Rats After Oral Administration

Male ZDF rats were randomly and evenly divided into two groups of four animals each. The first group (treatment) was administered with 1 ml of the log-growth-phase LL-pUBGLP-1 suspension (10^9 cfu/ml) in PBS. The second group (control) was administered with GLP-1 solution (1000 nmoles/kg) in 1 ml of the log-growth-phase LL-pUB1000 suspension (10^9 cfu/ml). The animals in each group were fasted for 4 h with free access to water before the oral dosing. Fasting was continued until the completion of the study. The blood samples were collected at 45 min, 30 min and 15 min pre-dose and at 0, 1, 2, 4, 6, 8, 11 h post-dose and subjected for immediate glucose measurement by an Ascencia® Contour glucometer and insulin assay by ELISA as described by manufacturer's protocol.

Data Analysis

Statistical analysis was performed using Student *t*-test with $\alpha = 0.05$ as the minimal level of significance. Wherever possible, the data are presented as mean ± standard deviation. Analysis was performed with Microsoft® Excel 2003.

RESULTS

LL-pUBGLP-1 Construction

Figure 2 displays the growth curve of the transformed *L. lactis*, LL-pUBGLP-1, and its secretion of rGLP-1. The growth of LL-pUBGLP-1exhibited the typical profile of bacteria growth in culture media: an initial slow-growing phase followed by a log-expanding phase and then a stationary phase. The secretion of rGLP-1 by LL-pUBGLP-1 was consistent with the growth profile. The total secretion of rGLP-1 in a 12-h period was 60 pM by an initial 10^4 cfu/ml of LL-pUBGLP-1in 500 ml culture media.

Insulinotropic Effect on HIT-T15 Cells

The secreted rGLP-1 by LL-pUBGLP-1 was tested for the ability to stimulate the secretion of insulin from pancreatic beta cells using HIT-T15 cells ($n=6$). As observed in Fig. 3, ~10^{10} cfu of LL-pUBGLP-1 stimulated an insulin secretion of 0.039±0.003 μg/mg of total protein, which was 190% of that

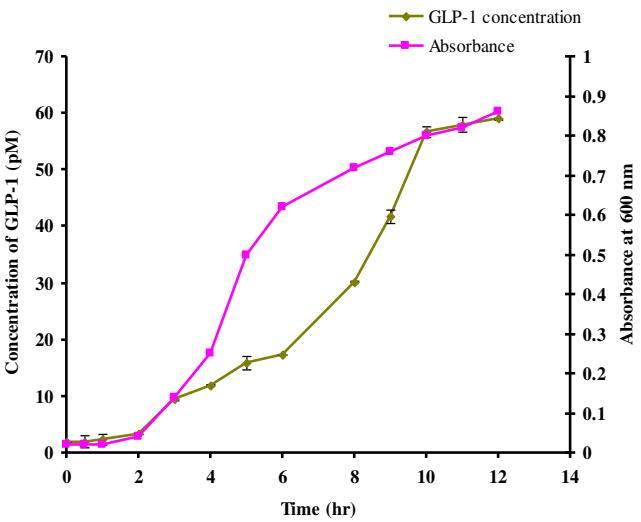

Fig. 2 Growth and GLP-1 secretion profile of LL-pUBGLP-1 in M17G medium.

by the negative controls (M17 and LL-pUB1000), $p<0.05$. The presence of 5 mM glucose significantly ($p<0.05$) increased the insulinotropic effect of LL-pUBGLP-1 (0.045± 0.001 μg/mg of total protein), but had no effect on the negative controls. The GLP-1 standard solution also exhibited a glucose-dependent insulinotropic effect. The insulin secretion stimulated by 50 pM GLP-1 solution was 0.042± 0.002 μg/mg of total protein, and increased to 0.049± 0.002 μg/mg of total protein in the presence of 5 mM glucose. Thus, it can be concluded that secretedrGLP-1 is bioactive and retained glucose-dependent insulinotropic behavior.

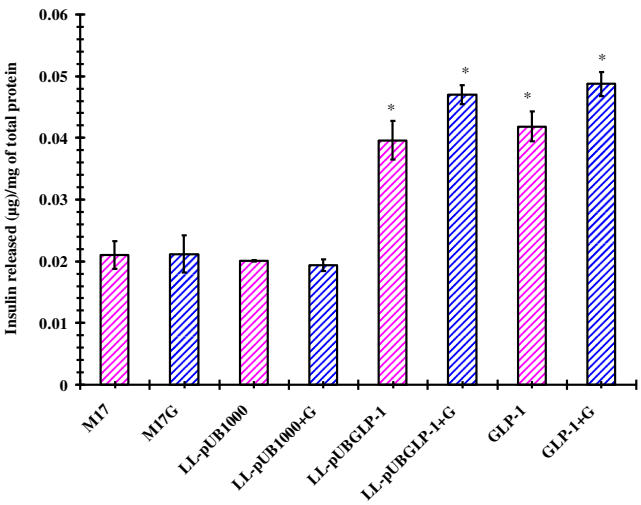

Fig. 3 Insulin release by HIT-T15 cells in presence of M17 media, M17 media + 5 mM Glucose (M17G), LL-pUB1000, LL-pUB1000 + 5 mM Glucose (LL-pUB1000 + G), LL-pUBGLP-1, LL-pUBGLP-1 + 5 mM Glucose (LL-pUBGLP-1 + G), GLP-1 solution, and GLP-1 + 5 mM Glucose (GLP-1 + G) (mean ± S.D, n = 6).*: significant difference from the control (M17 media), $p<0.05$.

MDCK Cell Viability in Presence of M17G

In order to culture LL-pUBGLP-1and MDCK cells together for the transport studies, selection of a common media where the growth of both LL-pUBGLP-1 and MDCK cells are not inhibited is required. Regular media for MDCK cells and LL-pUBGLP-1 are *s*-DMEM and M17G, respectively. This study was carried out to investigate the effect of M17G media on the viability of MDCK cells. The viability of MDCK cells in M17G media and s-DMEM (positive control) was carried out for 3 and 6 h period using MTT assay. The viability of the MDCK cells when incubated with M17G medium was close to 100% with respect to s-DMEM. Therefore, all further studies were carried out in M17G medium.

MDCK Cell Viability in Presence of LL-pUBGLP-1

The effect of LL-pUBGLP-1on the viability of MDCK monolayer was analyzed with trypan blue assay. The cell viability of the MDCK monolayer in presence of LL-pUBGLP-1 was found to be 99.21 (±1.05% and 97.95 (±1.82)% at 3 and 6 h respectively. There was no statistically significant difference ($p > 0.05$) in the cell viability in the presence or absence LL-pUBGLP-1. Thus LL-pUBGLP-1 has no deleterious effect on MDCK cell viability within the study time period.

MDCK Cell Monolayer Integrity in Presence of LL-pUBGLP-1

Transepithelial electric resistance (TEER) study was carried out to investigate the effect of LL-pUBGLP-1 on the MDCK cell monolayer integrity. The TEER values were monitored throughout the transportation studies and are shown in Fig. 4. There was no change in the TEER values in the presence of GLP-1 solution indicating that the cell monolayer integrity was not compromised during the experimental period. However, in the presence of LL-pUBGLP-1 there was a significant increase in the TEER value. This enhanced TEER value can be attributed to the adhesion of bacteria to the cell monolayer which will form another barrier for the flow of current. No clear correlation can be drawn between the absorption and TEER value. Previous studies in our lab have shown that TEER values cannot be used alone to ensure the integrity of the monolayer (41). Thus, mannitol and propranolol were also used for the same purpose.

The potential effect of LL-pUBGLP-1 on the integrity of MDCK cell monolayer was also examined by d-mannitol-[1-^{3}H(N)] and dl-propranolol-[4-^{3}H] hydrochloride as paracellular and transcellular transport markers respectively. The results for ^{3}H-mannitol transport are shown in Fig. 5a. Transport of ^{3}H-mannitol after 2 h in the presence LL-pUBGLP-1 was increased significantly ($p < 0.05$) as compared to the control (absence of LL-pUBGLP-1). Mannitol is transported through the paracellular route (42), thus, the enhanced transport in the presence of LL-pUBGLP-1 may be due to the opening of the tight junctions of the MDCK cell monolayer. These results are consistent with the previously published results where the transport of mannitol was enhanced in the presence of *L. lactis* (14). In contrast, the transport of propranolol was decreased in the presence of LL-pUBGLP-1 (Fig. 5b). It was found that there was no statistically significant difference in the total percentage of ^{3}H-propranolol transported between the two groups ($p > 0.05$) in a 5 h period. No significant increase in the percentage transport of

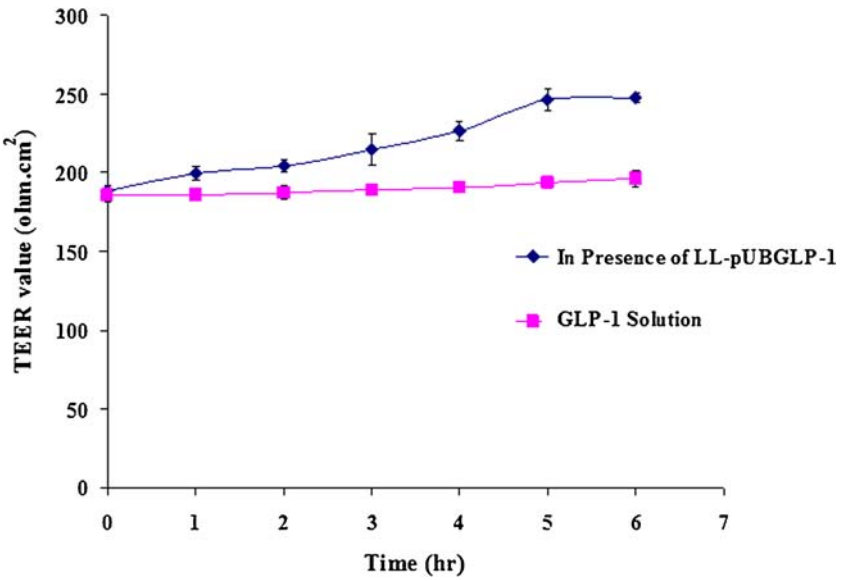

Fig. 4 Transepithelial electric resistance of the MDCK cell monolayer in the presence of LL-pUBGLP-1 and plain GLP-1 solution during the transport study (mean ± S.D., $n = 6$).

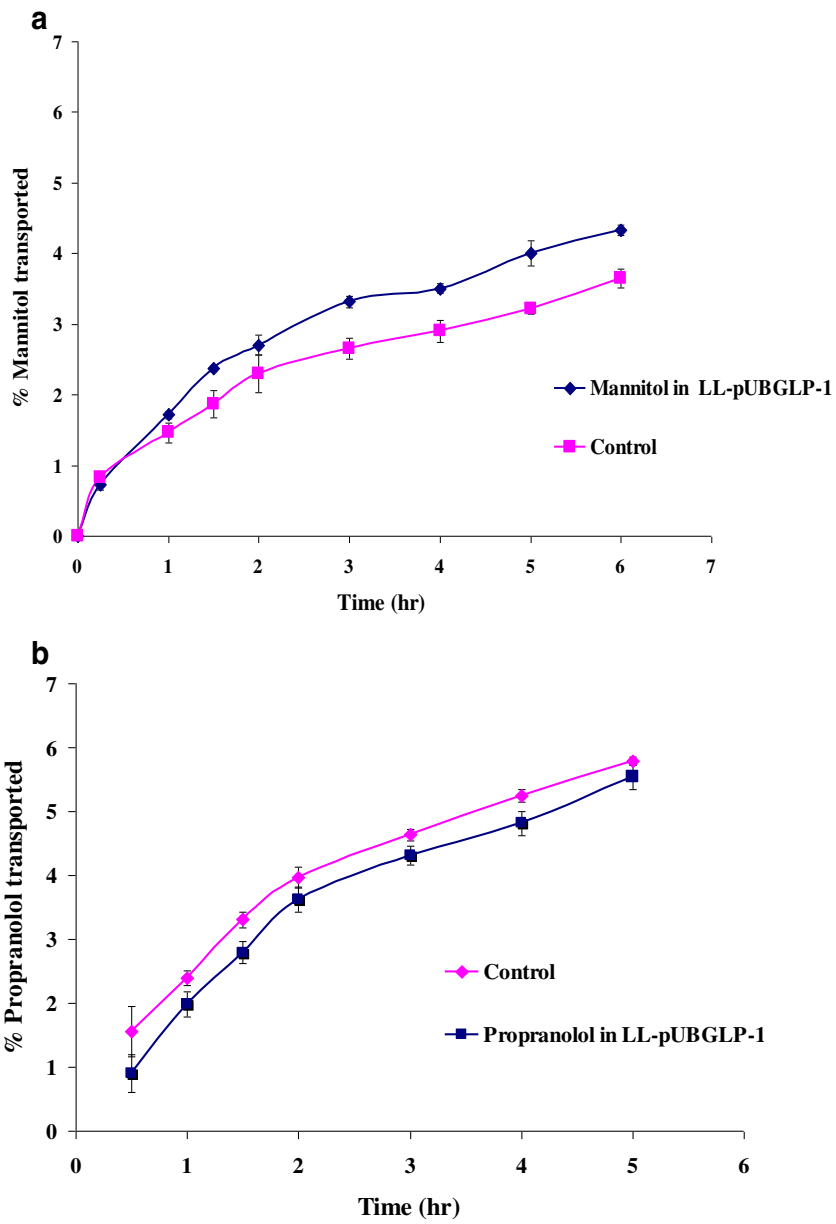

Fig. 5 **(a)** Effect of *L. lactis* on the transport of ^{3}H-mannitol across the MDCK cell monolayer. To the apical side of the confluent MDCK monolayer in each well, 1.5 ml of 66.7 nM ^{3}H-mannitol was added with LL-pUBGLP-1 or in the absence of LL-pUBGLP-1 (control) in M17G at time zero. Samples (500 μl) were withdrawn from the basal side at different time points for the mannitol assay (mean ± S.D., $n = 6$). (**b**) Effect of LL-pUBGLP-1 on the transport of ^{3}H-propranolol across the MDCK cell monolayer. To the apical side of the confluent MDCK monolayer in each well, 1.5 ml of 35.5 nM ^{3}H-propranolol was added with LL-pUBGLP-1 or in the absence of LL-pUBGLP-1 (control) in M17G at time zero. Samples (500 μl) were withdrawn from the basal side at different time points for the propranolol assay (mean ± S.D., $n = 6$).

propranolol in the presence of LL-pUBGLP-1 indicates that the MDCK cell monolayer integrity (transcellular route) was maintained throughout the studies.

Delivery of rGLP-1 Through MDCK Monolayer by LL-pUBGLP-1

Next, LL-pUBGLP-1 was investigated for the ability to enhance the absorption of rGLP-1 through the MDCK monolayer and the results are presented in Fig. 6. Since the concentration of rGLP-1 on the apical side was different for different wells and also varied with time it was not possible to calculate the apparent permeability of rGLP-1. Therefore, to compare the transport rate of rGLP-1, AUC $_{0\text{-}6h}$ was used as representative of total drug exposure on the apical side of the monolayer. AUC_{0-6h} was calculated by linear trapezoid method. The transport rate was normalized by this AUC $_{0\text{-}6h}$ according to the following equation:

$$\text{Normalized transport rate} = \frac{X}{AUC_{0-6h}}$$

Where, X = cumulative amount of GLP-1 found in the basal side of the cell monolayer during 6 h period

And AUC = total amount-time curve at the apical side, calculated using trapezoidal method.

The transport rate was 0.32 ± 0.07 h^{-1} and 0.037 ± 0.15 $h^{-1}$when delivered by the LL-pUBGLP-1 and the free solution form, respectively. In the presence of LL-pUB1000, the transport rate of the free solution did not increase. Therefore,

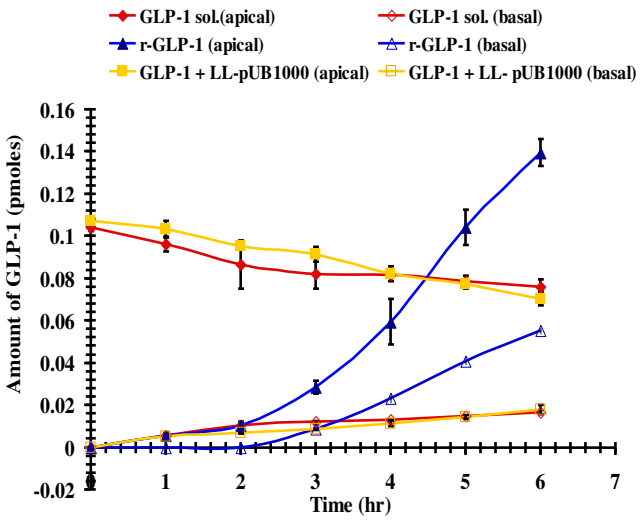

Fig. 6 Transport of GLP-1 across MDCK cell monolayer. Apical concentration of GLP-1 solution (*black diamond*); basal concentration of GLP-1 solution (*white diamond*); apical concentration of GLP-1 solution along with LL-pUB1000 (*black square*); basal concentration of GLP-1 solution transported in the presence of LL-pUB1000 (*white square*); apical concentration of r-GLP-1 secreted from LL-pUBGLP-1 (*balck triangle*); basolateral concentration of r-GLP-1 (*white triangle*). (mean ± S.D., $n = 6$).

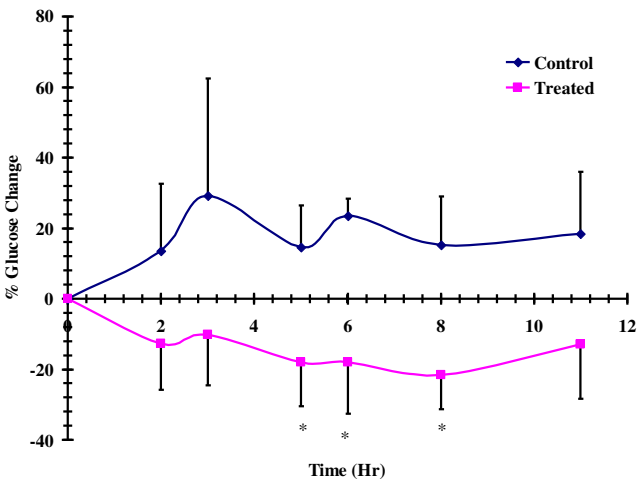

Fig. 7 Blood glucose level after oral administration of 10^9 cfu of LL-pUBGLP-1 (*black square*) and GLP-1 solution 1000 nmoles/kg (*black diamond*) in ZDF rats (mean ± S.D, $n = 4$).*: significant difference from the GLP-1 solution-treated group, $p < 0.05$.

LL-pUBGLP-1 demonstrated significantly enhanced transport in comparison with the free solution form ($p < 0.01$). This enhanced delivery is most probably due to concentration of rGLP-1 on the absorption surface (the MDCK monolayer surface) at the donor side since the LL-pUBGLP-1 adhered to the monolayer and secreted the rGLP-1 directly onto it.

Pharmacodynamic Effects of LL-pUBGLP-1 in ZDF Rats After Oral Administration

The T2DM treatment efficacy by the LL-pUBGLP-1 after oral administration was investigated in ZDF rats (Fig. 7). In the treated group, the blood glucose level decreased by 10–20% 2–12 h after the oral administration of 10^9 cfu/ml of LL-pUBGLP-1. However, in the control group, the oral administration of 1000 nmoles of GLP-1 per kg body weight plus 10^9 cfu/ml of LL-pUB1000, no decrease in the blood glucose levels from the baseline throughout the course of the study were observed. Compared to the control group, there was a significant decrease ($p < 0.05$) in blood glucose concentration by the LL-pUBGLP-1 at 5, 6 and 8 h post-dosing.

Plasma insulin levels were also monitored in the treated and control groups (Fig. 8). While the control group showed a steady insulin plasma level around 1–2 μg/l during the 11 h study period, the oral dosing of LL-pUBGLP-1 resulted in an insulin peak of 8 μg/l in plasma at 5 h. The insulin AUC_{0-11} of the LL-pUBGLP-1 group was 2.5 times of that in the control group ($p < 0.01$). Therefore, both the glucose level and insulin concentration in plasma demonstrate the effectiveness of LL-pUBGLP-1 in Type-2 diabetic rat models, indicating the rGLP-1 secreted by LL-pUBGLP-1 was adequately absorbed to exert its pharmacological effect.

DISCUSSION

Since the discovery of insulin as a first protein drug, several protein drugs became available that played a critical role in the management of T2DM. GLP-1 analogue is one of the newest classes of drugs for the management of T2DM. Despite the recent advancement in GLP-1 based therapy such as long acting analogues, which require weekly administration by subcutaneous route, injection still remains the most

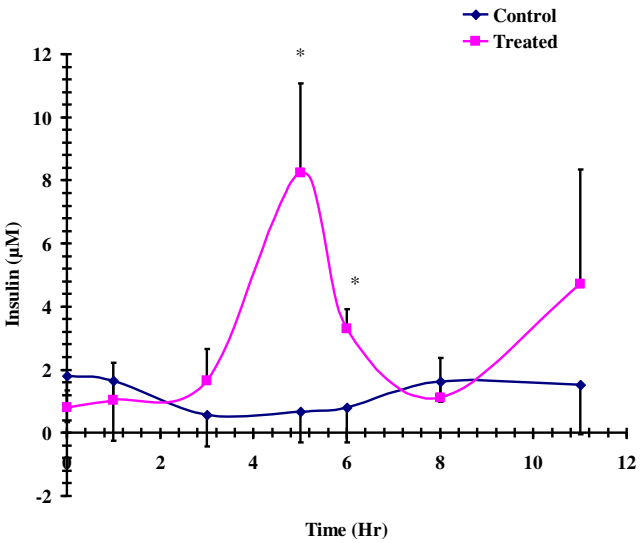

Fig. 8 Insulin concentration in the plasma after oral administration of 10^9 cfu of LL-pUBGLP-1 (*squares*) and GLP-1 solution 1000 nmoles/kg (*diamonds*) in ZDF rats (mean ± S.D, $n = 4$). *: significant difference from the GLP-1 solution-treated group, $p < 0.05$.

common method for the administration of such peptide drugs. One of the major disadvantages of injectables is inconvenience and poor patient compliance during chronic treatment for disease such as T2DM. Oral route of drug delivery is preferred to any other route because of its high levels of patient acceptance and long term compliance, which eventually results in increasing the therapeutic value of the drug. Therefore, there is an imperative need to develop a delivery system that can deliver GLP-1 orally. Several publications in the past have attempted to use lactic acid bacteria primarily for the mucosal delivery of drug molecules (43). In this work we have extended the use of lactic acid bacteria to systemically deliver peptide drugs through oral route. The present work aims to develop recombinant *L. lactis* as an oral delivery system for the delivery of GLP-1. To achieve this goal, the bacteria must be first engineered to be able to produce and secrete rGLP-1. Second, the rGLP-1 should retain the biological activities. Third, the system should be effective *in vivo*.

In this study, LL-pUBGLP-1 was constructed using pUB1000 as a vector backbone. DNA sequencing confirmed the successful construction of LL-pUB1000. *In vitro* growth curve of recombinant *L. lactis* along with the secretion study in the culture media showed that the LL-pUBGLP-1 could secrete rGLP-1 (Fig. 2). Incretins, such as GLP-1 is shown to exert its pharmacodynamics effect through enhanced glucose dependent insulin secretion. In this study, a well-known pancreatic β-cell model HIT-T15 was used to test the biological activity of secreted rGLP-1 from LL-pUBGLP-1.

Co-incubation of the HIT-T15 cells with the LL-pUBGLP-1 bacteria led to a significant increase in insulin concentration in the culture media. These results demonstrated that the rGLP-1 secreted by the LL-pUBGLP-1 was able to stimulate the beta cells to secrete insulin (Fig. 3). In addition, biological activity of rGLP-1 was further confirmed by enhanced insulin secretion in the presence of glucose. After the delivery system was constructed and shown to release active form of GLP-1, we further explored the possibility of using this system in *in vitro* and *in vivo* settings. The MDCK cell line, a widely accepted model for oral absorption assessment, was (44) selected as the cell culture model for the *in vitro* transport of secreted GLP-1. A significant increase in the transport rate of rGLP-1 was observed as compared to the GLP-1 solution in the presence of LL-pUB1000 (eight-fold, $p<0.05$) (Fig. 6). This increase in the transport of rGLP-1 by LL-pUBGLP-1 delivery could be because of three possible reasons: 1) damage to the cell monolayer in the presence of the bacteria, 2) opening of tight junctions of the monolayer by the bacteria, or 3) concentrated deposition of r-GLP-1 on the apical surface of the monolayer and thus enhanced transport of GLP-1. The first possibility of compromised cell monolayer integrity was ruled out in the study where transcellular transport marker, propranolol, and the trans-epithelial electrical resistance measurement showed no difference in the presence of recombinant bacteria (Figs. 4 and 5b).

Increase in transport of paracellular marker mannitol, indicates that there is some opening of the tight junction of the cell monolayer. However, in the transport study LL-pUB1000 co-incubated with GLP-1 solution did not show any increase in the transport of GLP-1 across the cell monolayer. This observation could be possibly due to hindrance of GLP-1 transport by the bacterial layer. Therefore, these results indicate that this enhanced transport of rGLP-1 when delivered by LL-pUBGLP-1 could be either due to concentrated deposition on the absorption surface or the slight opening of the tight junctions. The exact mechanism of increased transport of rGLP-1 when delivered by LL-pUBGLP-1 needs to be further investigated.

Oral administration of LL-pUBGLP-1, in the ZDF rats a well-known non clinical model for Type II diabetes, showed a significant increase in insulin secretion and decrease in the blood glucose levels. In contrast, oral dosing of GLP-1 solution did not result in any increase in insulin secretion and showed an increase in blood glucose level. The reasons for increase in blood glucose level in the GLP-1 solution dosing group could be possibly attributed to stress. Further controlled studies will be conducted in future to understand the exact mechanism of action of recombinant bacteria. The pharmacodynamic effect seen with the LL-pUBGLP-1 suggests enhanced absorption of rGLP-1 in the systemic circulation. The observed pharmacodynamic effect in the treated group may have been underestimated due to stress related increase in blood glucose level as seen with the control group.

Thus, *in vivo* results in conjunction with the *in vitro* results demonstrate that the recombinant LL-pUBGLP-1 has the ability to secrete active rGLP-1 that can be sufficiently absorbed to exert its pharmacological effects. In addition, the effect of the reduction of glucose level lasted for the entire study period of 11 h, indicating that rGLP-1 was continuously secreted by the LL-pUBGLP-1 and absorbed over this time period. Also, the maximum glucose lowering effect was observed during exponential phase of bacterial growth, which was seen around 5–8 h in this study. This along with previous published work suggests the feasibility of delivery of proteins and peptides using recombinant lactic acid bacteria (18,32,33).

This approach can be extended to other protein and peptide drugs and may result in several advantages over the conventional methods for peptide drug delivery. First, it provides a feasible and efficient approach for non-invasive oral delivery of protein drugs. When the protein drugs are given orally by conventional dosage forms such as tablets or solutions, they are extensively digested or degraded before reaching the absorption epithelium or before being absorbed. Thus, the minimum amounts present on the absorption surface together with the low permeability nature usually result in

negligible absorption. Many non-invasive routes have also been tried and studied extensively both in academic and industrial laboratories with varying degree of success. Major efforts have been directed towards developing effective oral formulations and increasing the oral absorption of intact protein through the use of formulations that protect the macromolecule and/or enhance its uptake into the intestinal mucosa. However, in spite of remarkable attempts, relatively little progress has been made (45–47).

The second advantage that the transformed normal flora can provide is prolonged delivery. After entering the intestine, the bacteria will temporarily colonize the intestine mucosa and proliferate, and then gradually progress down the digestive system. These bacteria may remain in the intestine for several days, depending on their adhesion properties, the dose administered, and other normal flora that are both pre-existing and taken in later. Therefore, the medicated normal flora have the potential to continuously produce and deliver the drug for a period of several days, which is desired for many cases where either the drug has a short half-life or chronic treatment is required.

Although this work along with previously published work (18) provides a novel strategy to orally deliver protein drugs there are several issue that need to be addressed before this can be used in clinical practice. Further studies needed to be investigated are i) the fate of recombinant bacteria after oral administration, ii) the impact of gastrointestinal environment on the growth and bacterial survival, iii) the exact mechanism of enhanced absorption of protein drug, iv) formulation strategies such as enteric coated formulations to protect bacteria from harsh gastrointestinal environment, v) optimization of the bacterial dose to maintain adequate blood glucose levels, and vi) potential environmental pollution by the recombinant bacteria after being eliminated out of the body.

CONCLUSION

A recombinant *L. lactis* has been developed for the oral delivery of GLP-1 and demonstrated to be effective in rats with T2DM, indicating that normal flora can be utilized as an effective vehicle for oral delivery of some protein drugs.

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