RESEARCH ARTICLE

Heterologous Expression and Delivery of Biologically Active Exendin-4 by *Lactobacillus paracasei* L14

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Abstract

Exendin-4, a glucagon-like protein-1 (GLP-1) receptor agonist, is an excellent therapeutic peptide drug for type 2 diabetes due to longer lasting biological activity compared to GLP-1. This study explored the feasibility of using probiotic *Lactobacillus paracasei* as an oral vector for recombinant exendin-4 peptide delivery, an alternative to costly chemical synthesis and inconvenient administration by injection. *L. paracasei* transformed with a plasmid encoding the exendin-4 gene (*L. paracasei* L14/pMG76e-exendin-4) with a constitutive promotor was successfully constructed and showed efficient secretion of exendin-4. The secreted exendin-4 significantly enhanced insulin secretion of INS-1 β-cells, along with an increment in their proliferation and inhibition of their apoptosis, corresponding to the effect of GLP-1 on these cells. The transcription level of the pancreatic duodenal homeobox-1 gene (*PDX*-1), a key transcription factor for cellular insulin synthesis and secretion, was upregulated by the treatment with secreted exendin-4, paralleling the upregulation of insulin gene expression. Caco-2 cell monolayer permeability assay showed a 34-fold increase in the transport of exendin-4 delivered by *L. paracasei* vs. that of free exendin-4 (control), suggesting effective facilitation of exendin-4 transport across the intestinal barrier by this delivery system. This study demonstrates that the probiotic *Lactobacillus* can be engineered to secrete bioactive exendin-4 and facilitate its transport through the intestinal barrier, providing a novel strategy for oral exendin-4 delivery using this lactic acid bacterium.

Introduction

Glucagon-like peptide-1 (GLP-1) is a potent incretin produced by L-cells of the distal ileum and colon in response to nutrient intake [1]. It has a variety of antidiabetic effects that are mediated through β-cells in pancreatic islets and peripheral tissues. These include not only the

promotion of glucose-dependent insulin secretion and inhibition of glucagon secretion [2, 3], but also inhibition of food intake and body weight gain by delaying gastric emptying [3], increasing β-cell mass [4, 5], and enhancing insulin sensitivity [6, 7]. However, despite its favorable antidiabetic functions, the native form of GLP-1 is unsuitable for therapeutic use because of its extremely short half-life *in vivo* (~1–2 min), due to its rapid inactivation by the ubiquitous enzyme dipeptidyl peptidase IV (DPP-IV) [8]. Two GLP-1-based strategies have been adopted to solve this problem: DPP-IV-resistant GLP-1 analogs and DPP-IV inhibitors [9]. Sitagliptin, saxagliptin and vildagliptin are examples of currently available DPP-IV inhibitors. Their major advantage is that they can be administered orally; however, many other hormones are also substrates of DPP-IV, and DPP-IV inhibition may affect the pathways mediated by those hormones [10].

Exendin-4, the first discovered GLP-1 mimetic (with 53% homology), was originally isolated from the salivary secretions of the Gila monster *Heloderma suspectum* [11]. Exendin-4 binds to and activates the GLP-1 receptor, and thus shares many antidiabetic actions with GLP-1, such as glucose-dependent increase of insulin secretion, suppression of glucagon secretion, reduction of food intake and facilitation of β-cell neogenesis [12–15]. Exendin-4 has a longer biological half-life than GLP-1 due to its resistance to DPP-IV degradation, and can serve as a potent and longer-acting agonist of the pancreatic GLP-1 receptor [16]. Synthetic exendin-4 (exenatide) was approved as the first GLP-1 receptor agonist for diabetes treatment by the US Food and Drug Administration (FDA) in 2005. Today, an exenatide extended-release suspension (Bydureon) is available, administered by weekly injection [17].

Oral administration is the preferred method of drug delivery due to patient acceptance, long-term compliance and few side effects [18, 19]. However, this strategy is usually not feasible for protein or peptide drugs due to poor bioactivity resulting from proteolysis during passage though the gastrointestinal environment and poor epithelial permeability [20]. Many approaches have been explored to increase the oral bioavailability of antidiabetic peptides by protecting them from enzymatic digestion and increasing their absorption across the intestinal wall [19, 20]. Some of the most widely studied strategies include the use of nanoparticles [20, 21], mucoadhesive polymers [22], absorption enhancers [23] and oral films [24]. Although these strategies have provided novel prospects for the problem, most of them have encountered roadblocks such as low efficacy and high toxicity, and have barely advanced to clinical trials [25].

In the past two decades, lactic acid bacteria (LAB) have been demonstrated as promising oral delivery vehicles for protein/peptide drugs [26–28]. Before that, LAB were used for centuries in traditional fermented products in the food industry; they are generally regarded as safe (GRAS) by the FDA. In addition, specific strains of LAB, and especially of *Lactobacillus*, have many intrinsic probiotic effects on human and animal health, such as maintaining gastrointestinal homeostasis and modulating immunity [29]. Furthermore, many *Lactobacillus* strains can survive transit through the human gastrointestinal tract and tightly adhere to the intestinal epithelial cells, facilitating mucosal targeting and transport of therapeutic molecules while minimizing bacterial and enzymatic degradation of the protein-based drugs [30, 31]. Because of these advantages, LAB have been regarded as an appealing potential alternative to other oral delivery systems. A study on the use of interleukin (IL)-10-transformed *Lactococcus lactis* to treat Crohn's disease has passed phase I clinical trials [32]. Several articles exploring the potential of transformed LAB for diabetes treatments have also been published, including the use of *L. lactis* to deliver insulin analog [33–35] or combined expression of IL-10 and glutamic acid decarboxylase (GAD65) [36], and the use of *Bifidobacterium longum* to deliver GLP-1 [37] or *Lactobacillus gasseri* to deliver GLP-1(1–37) [38]. Here, we evaluated whether *Lactobacillus paracasei* can secrete bioactive exendin-4, to provide an alternative route for the use of LAB as a vector for oral delivery of exendin-4.

In this study, a *L. paracasei* strain transformed for expression and secretion of bioactive exendin-4 was explored as a novel delivery system for this GLP-1 mimetic. The expression characteristics of exendin-4 in *L. paracasei* were assayed and the bioactivities of recombinant exendin-4 were evaluated on the *in vitro* pancreatic β-cell line INS-1 as a model. The transport of exendin-4 through intestinal epithelial cells was also assayed using Caco-2 cell monolayers *in vitro*.

Materials and Methods

Materials

The Caco-2 cell line and the rat insulinoma cell line INS-1were purchased from American Type Culture Collection. Dulbecco's modified eagle medium (DMEM), Roswell Park Memorial Institute medium (RPMI) 1640, fetal bovine serum (FBS), non-essential amino acids (100X), and 0.25% trypsin with EDTA were obtained from Gibco BRL Life Technology. Transwell® inserts, treated tissue culture (0.4 μm pore size, 4.7 cm^2 surface area) and culture flasks were purchased from Costar Corporation (Corning, NY).

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown at 37°C in Luria-Bertani (LB) medium with shaking at 200 rpm. *L. paracasei* L14 was aerobically propagated statically at 37°C in de Man, Rogosa and Sharpe (MRS) medium. Antibiotics were used at the following concentrations: 500 μg/ml erythromycin or 100 μg/ml ampicillin for *E. coli* and 5 μg/ml erythromycin for lactobacilli.

DNA manipulation and transformation

The *exendin-4* cDNA sequence was obtained from GenBank (DI206426.1) and was codon optimized for LAB using the Codon Adaptation Tool (JCAT). An oligonucleotide carrying a fusion of signal peptide Usp45 and LEISSTCDA propeptide sequences, *exendin-4*, 6×His tag and *Xba* I and *Xho* I cDNA restriction sites between Usp45 and the 6×His tag was synthesized by GEN-EWIZ Biological Technology Co. Ltd. (Suzhou, China). This was incorporated into pMG76e vector (derived from pMG36e [39]) to yield pMG76e-exendin-4. The pMG76e-exendin-4 and empty control pMG76e plasmids were transformed into competent *L. paracasei* L14 cells by electroporation.

Heterologous expression and secretion of exendin-4 by *L. paracasei* L14

Table 1. Bacterial strains and plasmids used in this study.

Strains/plasmids	Description	Source or reference
Strains		
Lactobacillus paracasei L14	Wild-type, isolated from milk	CGMCC NO. 4015
***L. paracasei* L14/pMG76e**	*L. paracasei* L14 harboring empty vector pMG76e	This study
***L. paracasei* L14/pMG76e-exendin-4**	*L. paracasei* L14 harboring pMG76e-exendin-4	This study
Plasmids		
pMG76e	Derivative of pMG36e, 3.5-kb, Emr, containing the P32 promoter	Laboratory stock
pMG76e-**exendin-4**	pMG76e carrying *exendin-4* gene	This study

doi:10.1371/journal.pone.0165130.t001

To detect exendin-4 expression, the recombinant *L. paracasei* L14/pMG76e-exendin-4 and control (*L. paracasei* L14/pMG76e) transformants were grown overnight in MRS medium supplemented with 5 μg/ml erythromycin. A 1% (v/v) inoculum of the *L. paracasei* transformants was transferred into 50 ml fresh MRS medium and incubated statically at 37°C for 12 h; 100 μl of culture supernatant was sampled every 2 h for ELISA assay of exendin-4 secretion.

To prepare exendin-4-conditioned medium for INS-1 cell experiments, RPMI 1640 medium was used for exendin-4 secretion (RPMI 1640–exendin-4 medium). Overnight cultures of *L. paracasei* L14/pMG76e-exendin-4 and *L. paracasei* L14/pMG76e were diluted 1:100 in fresh MRS medium and incubated at 37°C for 12 h, to a bacterial cell density of about 10^9 cfu/ml. Cells were then harvested and washed twice in PBS (pH 7.4) buffer, suspended in RPMI 1640 buffered with 20 mM HEPES (pH 7.4), and incubated for 12 h. The culture was centrifuged at $8000 \times g$ for 10 min at 4°C. The supernatant was collected, adjusted to pH 7.4 with sodium hydroxide, and sterilized by filtration through a 0.22-μm membrane as RPMI 1640–exendin-4 medium.

Recombinant exendin-4 extraction and western blot analysis

The transformed *L. paracasei* L14 strain cultures (10 ml of 10^9 or 10^{10} cfu/ml cells) were centrifuged at $6000 \times g$ for 10 min at 4°C. Soluble cytoplasmic and secreted protein fractions were respectively prepared. The cell pellets were washed three times with PBS, resuspended in 1 ml PBS buffer (pH 7.4) and ultrasonically disrupted. Cell debris were separated out by centrifugation and discarded. The supernatant was subjected to precipitation with trichloroacetic acid (10% w/v) for 2 h at 4°C; then the mixture was centrifuged at $12,000 \times g$ for 10 min, and the pellet was washed three times with ice-cold acetone and then dissolved in 50 μl PBS. Both cytoplasm and medium extract samples were mixed with 4× SDS loading buffer (12% w/v SDS, 6% v/v β-mercaptoethanol, 30% w/v glycerol, 0.05% w/v Coomassie blue G-250, 150 mM Tris-HCl pH 7.0), and subjected to Tricine SDS-PAGE (16% T, 6% C) analysis as described previously [40]. Proteins on the polyacrylamide gel were then semi-dry-electrotransferred to a PVDF membrane (Millipore). The 6×His-tagged exendin-4 proteins were analyzed by Western blotting with 6×His antibody (Cell Signaling Technology), horseradish peroxidase-conjugated goat anti-rabbit polyclonal IgG secondary antibody (Cell Signaling Technology) and the ECL Chemiluminiscent Substrate (Pierce) according to manufacturer's instructions.

Indirect ELISA quantification of secreted exendin-4

The concentration of exendin-4 secreted by recombinant *L. paracasei* was measured by indirect ELISA according to the method described by Zhang et al. [41] with some modifications. Standard exendin-4 (Sigma, 0.2 μg/ml) was used as the coated antigen. Equal volumes (100 μl) of monoclonal antibody against exendin-4 (Abcam) (1:4000) and standard exendin-4 (0–100 nmol/l) or culture supernatant samples of *L. paracasei* L14/pMG76e-exendin-4, preincubated overnight at 4°C, were applied to detect the captured exendin-4. Horseradish peroxidase-conjugated rabbit anti-mouse IgG (Abcam) (100 μl) was used at a 1:10,000 dilution and revealed by reaction with TMBS substrate (Amresco). Absorption was determined at 450 nm and 630 nm by Multiskan MK3 ELISA plate reader (Thermo Labsystems). The exendin-4 concentration was calculated by the standard curve method.

Cell culture of rat insulinoma cell line INS-1

INS-1 cells (passage 10–20) were cultured in RPMI 1640 medium containing 10% (v/v) FBS, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 10 mM HEPES, 100 U/ml penicillin and

100 µg/ml streptomycin, and cells were maintained at 37°C in a humidified (5% CO_2, 95% air) atmosphere.

Insulin secretion assay

INS-1 cells were seeded in 24-well plates at a density of 10^5 cells per well and grown to 100% confluence before the assay. The medium was changed 24 h before the assay. For the assay, cells were quickly rinsed in 1 ml Hank's balanced salt solution (HBSS) [42] with 3 mM glucose followed by a 2 h preincubation with 1 ml of the same buffer. Then the buffer was discarded, RPMI 1640–exendin-4 medium diluted 1:1 (v/v) with fresh medium plus 5 mM glucose (600 µl per well) were added, and the mixture was incubated for 2 h. Standard GLP-1 (100 nmol/l) in RPMI 1640 medium was used as a positive control. Preliminary experiments indicated that 5 mM glucose maximizes glucose-dependent insulin secretion of INS-1 cells stimulated by GLP-1 (S1 Fig). At the end of the incubation, supernatant was collected and centrifuged for 10 min at $4000 \times g$ at 4°C and insulin concentration was determined by ELISA kit (Millipore, St. Charles, MO, USA). The cells were collected to analyze mRNA expression of pancreatic duodenal homeobox-1 (*PDX*-1) and insulin gene (*Insulin*).

Quantitative reverse transcription PCR (qRT-PCR) analysis of expression of *PDX*-1 and *Insulin*

Total RNA was isolated from INS-1 cells using TRIzol Reagent (Invitrogen). Reverse transcription was performed using PrimeScript II first-strand cDNA synthesis kit (Takara, China), containing 1 µg of total RNA as the template. qRT-PCR was carried out using a SYBR Green assay kit (Tiangen). Specific primers for *PDX*-1, *Insulin* and the internal control gene β-actin (*Actb*) from *Rattus norvegicus* were designed with Primer Premier 5 software (Table 2). Gene expression was normalized by the $\Delta\Delta C_T$ method [43] using β-actin as the reference for the calculations. The experiment was carried out in triplicate and the average results are reported.

Cell-proliferation and apoptosis assay

INS-1 cells were seeded in 96-well plates at a density of 2×10^4 cells per well. After 36 h incubation, the cells were grown to nearly 60% confluence and the medium was replaced with RPMI 1640–exendin-4 medium diluted 1:1 (v/v) with fresh RPMI 1640 containing 10% FBS (100 µl per well). Standard GLP-1 in RPMI 1640 medium (100 nmol/l) was used as a positive control and pure RPMI 1640 medium was used as a blank control. The mixture was incubated for 24 h. The PI3K (phosphatidylinositol-3-kinase) antagonist treatment cells were preincubated with wortmannin (100 nmol/l) for 15 min before the treatment. The number of viable cells was then analyzed by cell counting kit-8 (CCK-8) assay (Biyuntian Biotech Co. Ltd.) based on the formation of formazan with sensitive changes in OD_{450} values. Briefly, at the end of the 24-h incubation, the medium was discarded and cells were washed twice in RPMI 1640 and incubated in a 100-µl mixture (with

Table 2. Primers used in this study.

Name	Sequence (5′→3′)	NCBI Gene ID of *Rattus norvegicus*
β-actin-F	CCACCCGCGAGTACAACC	*Actb*, Gene ID: 81822
β-actin-R	CCACGATGGAGGGGAAGAC	
PDX-1-F	GGTGCCAGAGTTCAGTGCTAA	*Pdx*-1, Gene ID: 18609
PDX-1-R	CCAGTCTCGGTTCCATTCG	
insulin-F	CCTGCCCAGGCTTTTGTCAA	Degenerate primers of *Insulin* (*Ins*1 and *Ins*2), Gene ID: 24505 & 24506, respectively
insulin-R	CCTCCAGTGCCAAGGTCTGAAG	

doi:10.1371/journal.pone.0165130.t002

90 μl RPMI 1640 and 10 μl CCK-8) in each well for 4 h. The absorbance was read at 450 nm using a Multiskan MK3 ELISA plate reader to determine the *in vitro* cell proliferation. For apoptosis assays, staurosporine (1 μmol/l) was added in all treatment groups for 1 h, after which the cells were treated with Annexin V-PI (propidium iodide) cell apoptosis kit (Biyuntian Biotech Co. Ltd.) and apoptosis was measured by flow cytometry using a FACScan (Becton Dickinson).

Transport of exendin-4 through Caco-2 monolayer

Caco-2 cells (10–20 passages) were cultivated in 25-cm^2 flasks in DMEM supplemented with 10% (v/v) FBS, 1% (v/v) non-essential amino acids and 100 IU/ml penicillin–100μg/ml strepto-mycin (s-DMEM) at 37°C in an atmosphere of 5% CO_2 and high humidity. Upon 80% conflu-ence, cells were harvested using 0.25% trypsin EDTA and seeded on the polyester inserts (0.4 μm pore size, 24 mm diameter, 4.67 cm^2 grown surface, Costar, Corning, NY) in 6-well transwell plates at a density of 10^5 cell/cm^2 and incubated under normal cell culture conditions. The culture medium was replaced every other day for the first week and then every day until it was ready for transport studies when the monolayer became confluent and the transepithelial electrical resistance (TEER) reading reached a plateau (about 21 days). The TEER was moni-tored according to the method described by Hubatsch et al [44].

After the Caco-2 monolayer cell became confluent, the culture medium was washed twice by PBS (pH 7.4). Then 1.5 ml of freshly made L14/pMG76e-exendin-4 cells in antibiotic-free s-DMEM (~10^8 cfu/ml) was added to the apical side and 2.6 ml of fresh s-DMEM without antibi-otics was added to the basolateral side. Caco-2 cell viability was then measured by trypan blue assay at 0, 2, 4 and 6 h after the addition of L14/pMG76e-exendin-4 cells. To assay exendin-4 transport through the Caco-2 monolayer, the plate was centrifuged at $45 \times g$ for 5 min after the bacterial cells were added and incubated under normal culture conditions. At predetermined time points, 100 and 200 μl supernatant samples were withdrawn from the apical and basolat-eral sides, respectively, for exendin-4 quantification by ELISA and replaced with an equal vol-ume of s-DMEM without the antibiotics. The normalized transport rate of exendin-4 was calculated according to Agarwal et al. [35], and TEER was monitored as well.

Statistical analysis

All experiments were performed in triplicate, and data were expressed as mean ± SD. All analy-ses were carried out by one-way ANOVA and significant differences ($P < 0.05$) between means were identified by Duncan's test using SPSS 20 (SPSS Inc.).

Results

Expression of recombinant exendin-4 in *L. paracasei* L14

Fig 1 shows the plasmid pMG76e-exendin-4 and the cDNA sequence of the codon-optimized exendin-4. The constructed plasmids pMG76e and pMG76e-exendin-4 were introduced into *L. paracasei* L14 and the resultant transformant culture and cells were prepared for the assays. Soluble cytoplasmic and secreted protein fractions were extracted from cells and culture media and subjected to Tricine SDS-PAGE and immunoblotting assays (Fig 2). Fig 2A shows the western blot analysis of recombinant exendin-4 of transformed strains cultured in MRS medium. A prominent 9.4-kDa band can be seen in the supernatants of both the bacterial cul-ture and the soluble cytoplasmic proteins from recombinant strain *L. paracasei* L14/pMG76e-exendin-4. This band matched the calculated molecular weight corresponding to the primary protein, containing the signal peptide Usp45 of the recombinant exendin-4. Thus, the signal peptide in the MRS supernatant could not be cleaved by *L. paracasei* signal peptidase.

RPMI 1640 medium buffered with 20 mM HEPES (pH 7.4) was conditioned with the transformant strain of *L. paracasei* L14/pMG76e-exendin-4 and used at different bacterial cell concentrations (10^9 cfu/ml and 10^{10} cfu/ml) to secrete exendin-4. The secreted exendin-4 showed multiple proteoforms (Fig 2B): a preprotein of 9.4 kDa was found in the soluble cytoplasmic fraction, and two protein bands, the 9.4-kDa preprotein and the mature 5.1-kDa protein with successful cleavage of the signal peptide, appeared in supernatants of the transformed strain. The bacteria at 10^{10} cfu/ml produced more recombinant exendin-4 than bacteria at 10^9 cfu/ml, indicating that exendin-4 expression and secretion levels are positively related to bacterial density. Prolonged incubation of the transformed *L. paracasei* strain in buffered RPMI 1640 medium produced mature exendin-4, implying that buffered RPMI 1640 medium may provide a more suitable biochemical environment for correct sorting and processing of secreted exendin-4 by the bacterial signal peptidases.

Fig 2C shows the growth curve of the transformed strain, *L. paracasei* L14/pMG76e-exendin-4, and its secretion of exendin-4. The secretion of exendin-4 by *L. paracasei* L14/pMG76e-exendin-4 was consistent with the bacterial growth profile. Total secretion of exendin-4 reached 28.5 nmol/l in 50 ml culture volume after 12 h incubation.

Recombinant exendin-4's insulinotropic effect in INS-1 cells

To determine whether the exendin-4 protein secreted by *L. paracasei* L14/pMG76e-exendin-4 retains its biological activity, its ability to stimulate insulin secretion was evaluated with rat pancreatic β-cell line INS-1. As observed in Fig 3, *L. paracasei* L14/pMG76e–exendin-4 medium significantly stimulated insulin secretion to 69.1 ± 0.3 ng/ml, which was 4% higher than the blank control (with only RPMI 1640, 66.5 ± 0.5 ng/ml, added) ($p < 0.05$), and 5% higher than the negative control (*L. paracasei* L14 with empty plasmid pMG76e-conditioned medium, 65.9 ± 0.8 ng/ml) ($p < 0.05$). Similarly prepared medium from *L. paracasei* L14 transformed with empty plasmid pMG76e failed to increase insulin secretion. The GLP-1 standard solution also displayed insulinotropic ability, which enhanced insulin secretion to 71.4 ± 0.7 ng/ml, 7% higher than the blank control ($p < 0.05$). Thus, it could be concluded that the *Lactobacillus*-secreted exendin-4 is bioactive and has an insulinotropic effect.

Recombinant exendin-4 increases mRNA expression of *PDX-1* and the *insulin* gene

PDX-1 is an important transcription factor, which plays an essential role in GLP-1-enhanced insulin transcription and synthesis, pancreas development and β-cell neogenesis [45–47]. GLP-
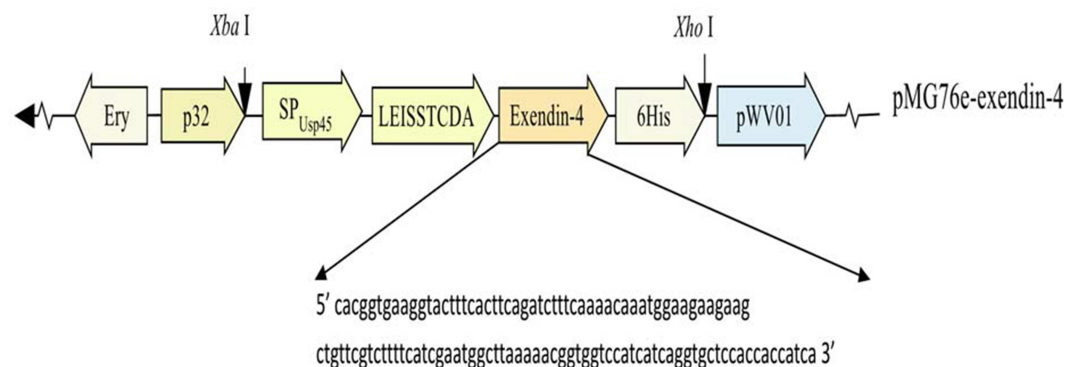

Fig 1. Expression cassette for recombinant exendin-4 secretion by *L. paracasei* strain L14. Expression of the recombinant exendin-4 was controlled by the constitutive p32 promoter. SP$_{Usp45}$: sequence encoding the signal peptide Usp45; LEISSTCDA: sequence encoding the synthetic LEISSTCDA propeptide; the codon-optimized cDNA sequence of exendin-4 is also supplied.
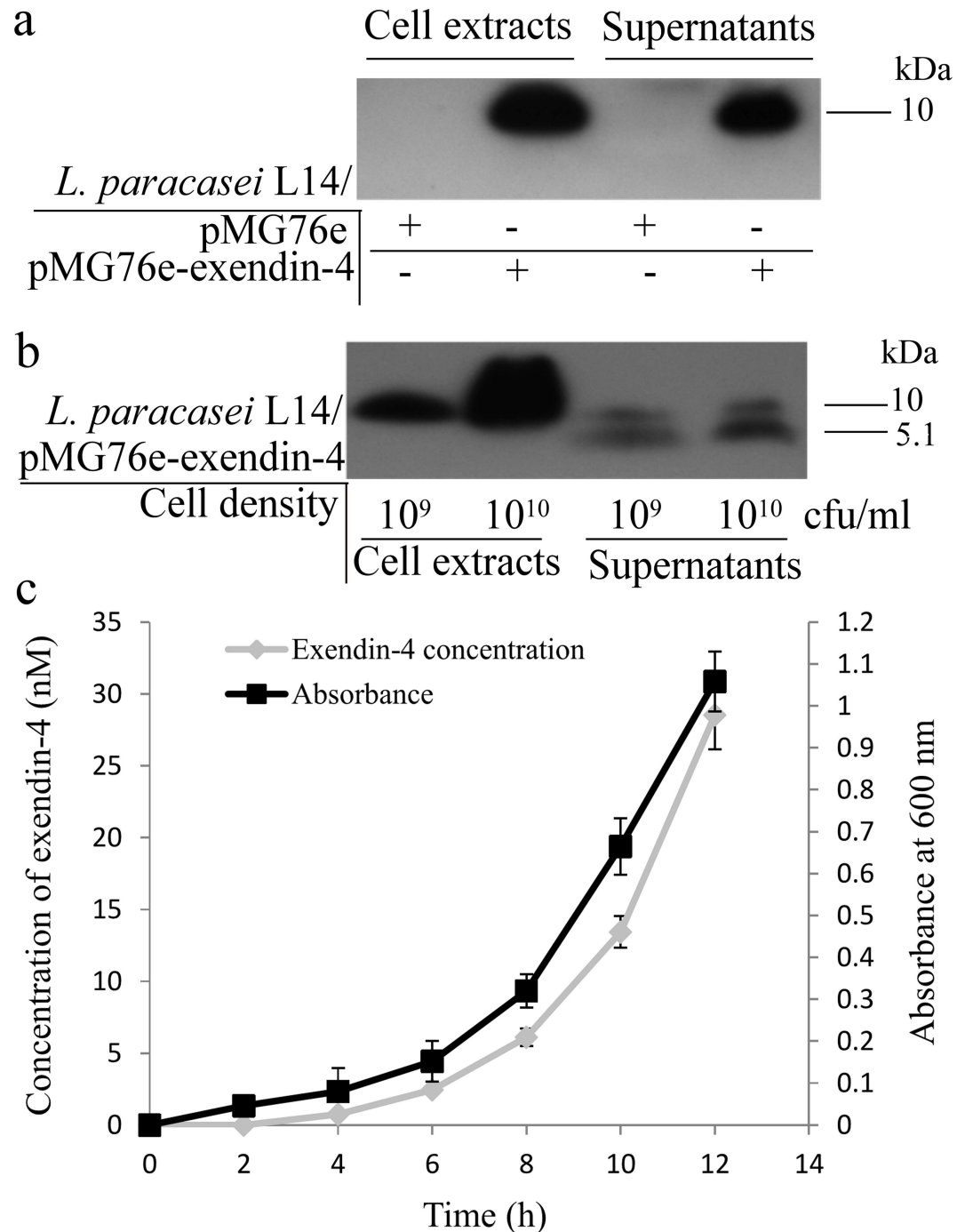
Fig 2. Western blotting and enzyme-linked immunosorbent assay (ELISA) analysis of recombinant exendin-4 produced by plasmid-transformed *L. paracasei* L14 strains. (a) Strains cultured in MRS medium: lanes 1 and 2, cell pellet of *L. paracasei* L14/pMG76e and *L. paracasei* L14/pMG76e-exendin-4 strains, respectively; lanes 3 and 4, excretion of *L. paracasei* L14/pMG76e and *L. paracasei* L14/pMG76e-exendin-4 strains, respectively. (b) Strains cultured in buffered RPMI 1640 medium: lanes 1 and 2, cell pellet extraction of *L. paracasei* L14/pMG76e-exendin-4 strain with bacterial concentration of 10^9 cfu/ml and 10^{10} cfu/ml, respectively; lanes 3 and 4, cell excretion of *L. paracasei* L14/pMG76e-exendin-4 strain with bacterial concentration of 10^9 cfu/ml and 10^{10} cfu/ml, respectively. (c) ELISA of exendin-4 expression *in vitro* at different time points of culture as described in the methods (three repeat expressions, means ± SD).
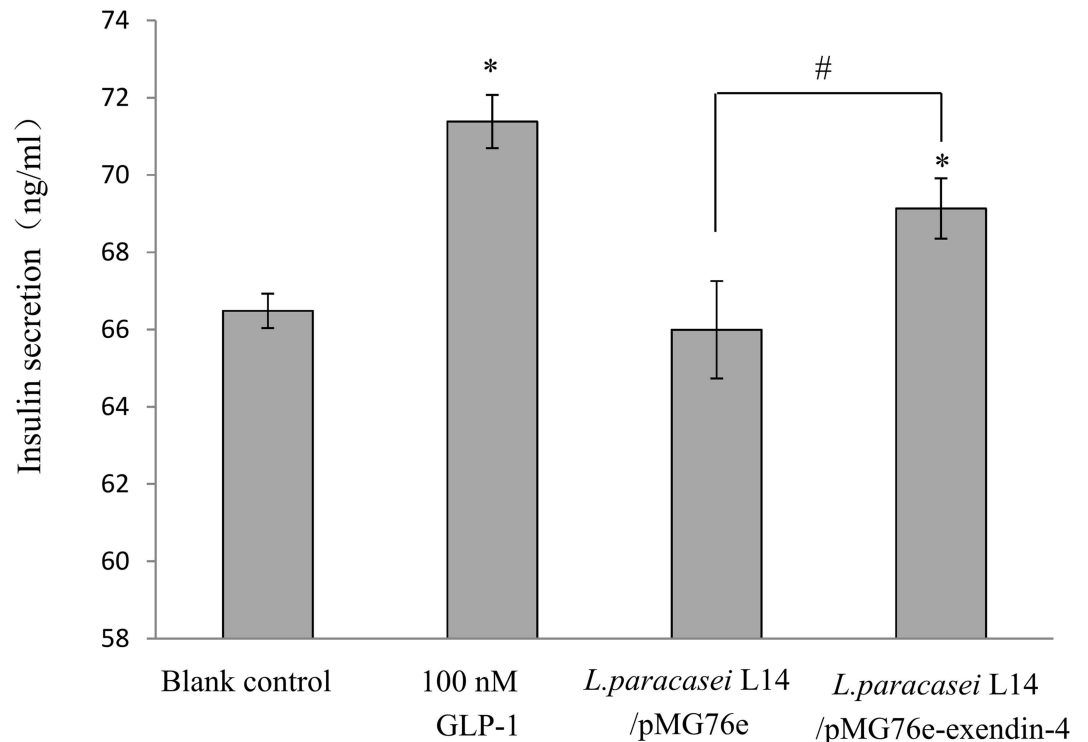
doi:10.1371/journal.pone.0165130.g002

Fig 3. Effect of recombinant exendin-4 on insulin secretion of INS-1 cells (means ± SD, n = 3). *$p < 0.05$ vs. control cells with pure cell culture media; ##$p < 0.01$ vs. *L. paracasei* L14/pMG76e-treated group.

doi:10.1371/journal.pone.0165130.g003

1 can increase pancreatic expression of PDX-1, enhancing the transcription and synthesis of insulin [47]. Thus, we also investigated mRNA expression levels of *PDX-1* and the *insulin* gene in INS-1 cells treated with RPMI 1640–exendin-4 medium for 2 h. As shown in Fig 4A, *L. paracasei* L14/pMG76e–exendin-4 medium significantly increased *PDX-1* mRNA expression ($p < 0.01$), which was 2.2-fold higher than in the blank control and 1.8-fold higher than in *L. paracasei* L14/pMG76e medium. The GLP-1 standard solution also increased *PDX-1* mRNA expression to 1.9-fold that of the blank control. *Insulin* mRNA expression was consistent with that of *PDX-1* (Fig 4B). *L. paracasei* L14/pMG76e–exendin-4 medium increased *insulin* mRNA expression 1.5-fold ($p < 0.01$) and 1.6-fold ($p < 0.01$) that of the blank control and *L. paracasei* L14 harboring empty plasmid, respectively. The standard GLP-1 also promoted *insulin* gene transcription ($p < 0.01$), which was 1.7-fold higher than the blank control. Taken together, these results suggested that the expression of *PDX-1* is upregulated by *L. paracasei* L14-secreted exendin-4, thereby exerting the functional effects of enhancing insulin transcription and synthesis.

Recombinant exendin-4 enhances INS-1 cell proliferation and decreases staurosporine-induced cell apoptosis

GLP-1, and its long-acting analog exendin-4, can also increase proliferation and decrease apoptosis of β-cells [4, 48]; both of these effects are prevented by treatment with wortmannin, a specific pharmacological inhibitor of PI3K [4]. Therefore, we determined whether the exendin-4 secreted by *L. paracasei* is also functionally involved in regulating proliferation and apoptosis of β-cells using the INS-1 cell model. As shown in Fig 5A, both conditioned media—with pMG76e and pMG76e-exendin-4 transformant culture—inhibited INS-1 cell growth compared to the blank control group. However, compared to pMG76e, pMG76e-exendin-4
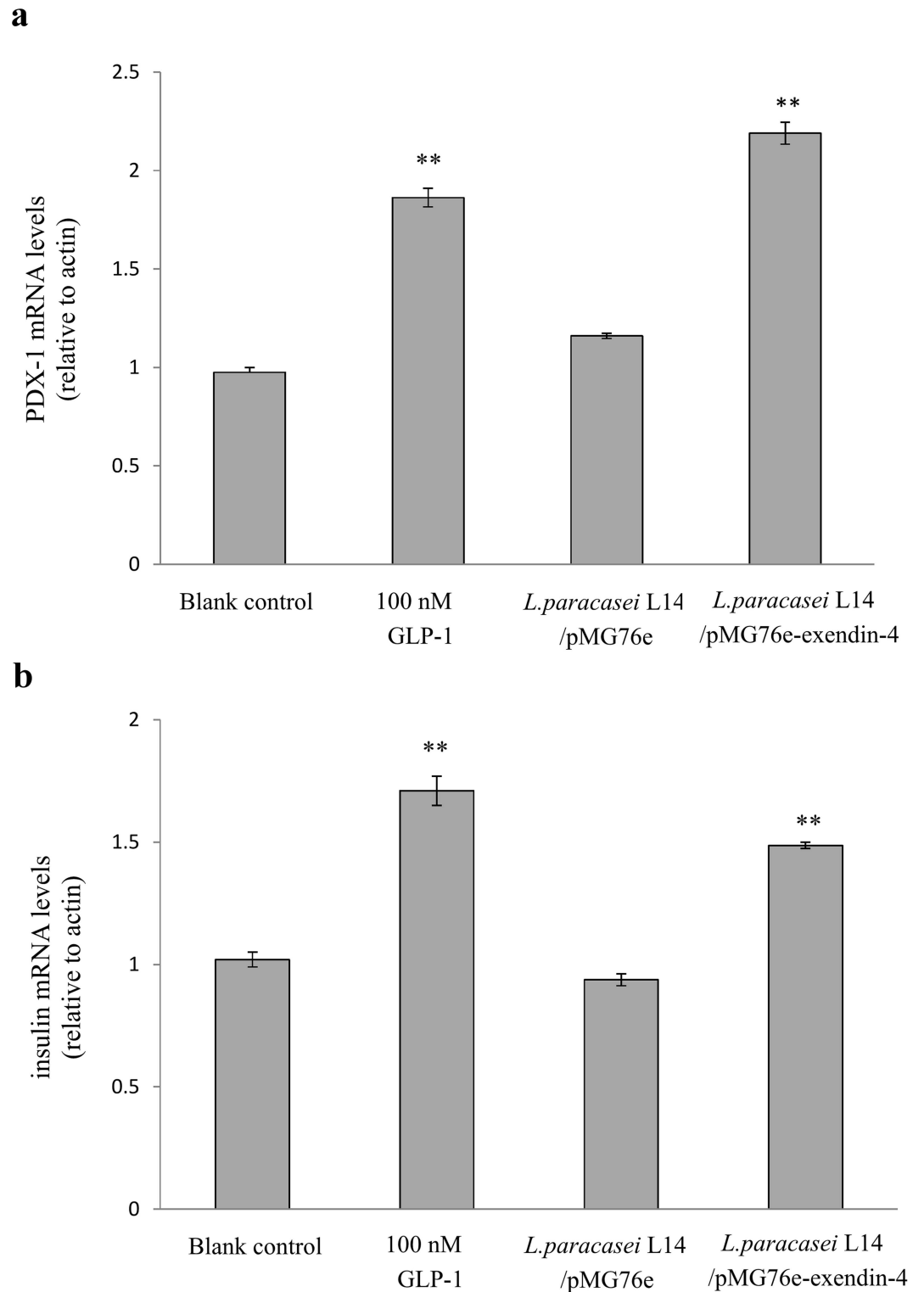
Fig 4. Effect of recombinant exendin-4 on the transcription of *PDX-1* and *insulin* gene in INS-1 cells. (a) *PDX*-1 mRNA expression level (means ± SD, n = 3). (b) *Insulin* mRNA expression level (means ± SD, n = 3). **$p < 0.05$ vs. control cells with pure cell culture media and *L. paracasei* L14/pMG76e-treated group.
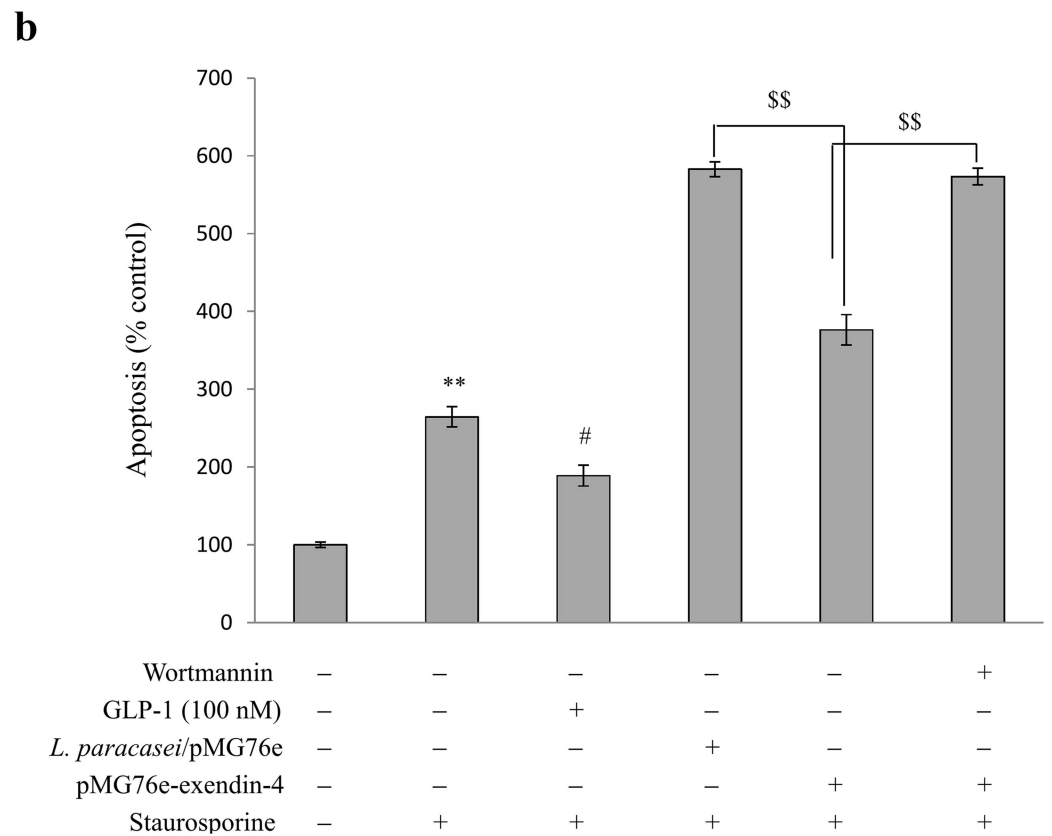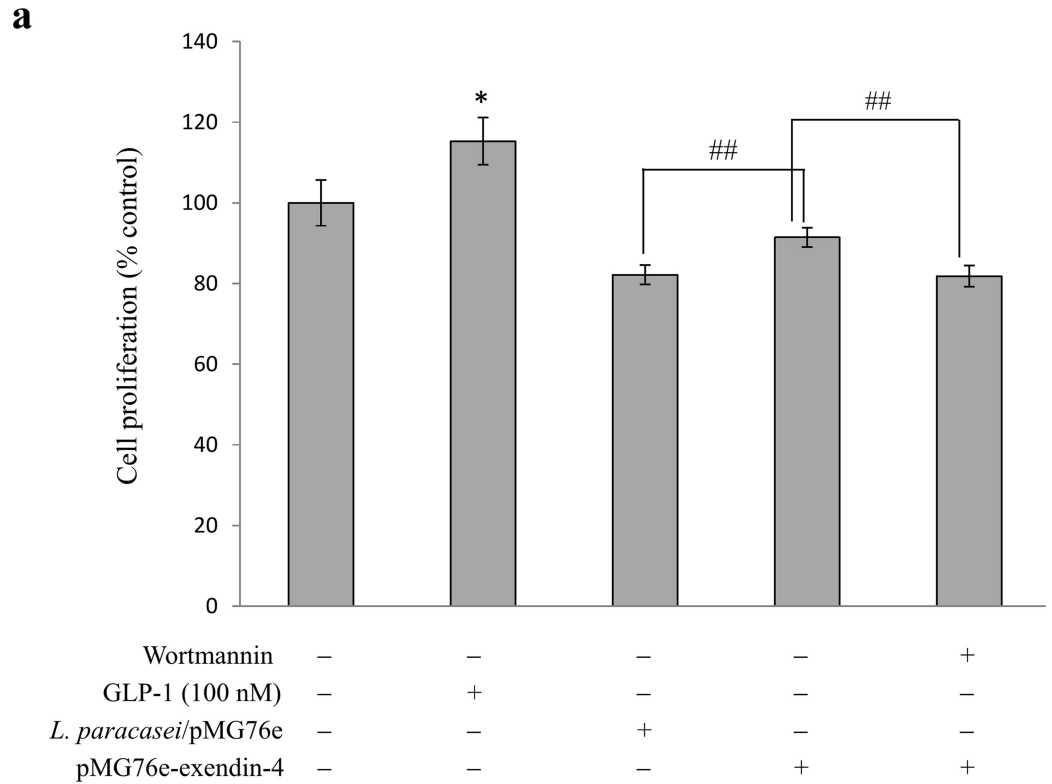
doi:10.1371/journal.pone.0165130.g004

Fig 5. Effect of recombinant exendin-4 on proliferation and staurosporine-induced apoptosis of INS-1 cells. (a) INS-1 cells were preincubated with media alone or with 100 nmol/l wortmannin, followed by treatment

with different agents as indicated for 18 h (means ± SD, n = 3). *$p < 0.05$ vs. control cells with pure cell media; ##$p < 0.01$. (b) INS-1 cells were preincubated with media alone or with 100 nmol/l wortmannin, followed by treatment with different agents as indicated for 18 h. Then cells were treated with media or 1 mmol/l staurosporine, as indicated (means ± SD, n = 3). **$p < 0.01$ vs. control cells with no staurosporine; #$p < 0.05$ vs. cells with only staurosporine; $^{\$\$}p < 0.01$.

transformant-conditioned medium significantly increased cell proliferation ($p < 0.01$), by 13.5%. On the other hand, when wortmannin was added, there was no significant difference in cell proliferation between the pMG76e and pMG76e-exendin-4 treatments, indicating that the recombinant exendin-4 improves INS-1 cell proliferation via the PI3K signal pathway. The positive control, 100 nmol/l standard GLP-1, also increased cell proliferation ($p < 0.05$), which was 15.3% higher than with the blank control.

After a 1 h treatment with staurosporine, a general kinase inhibitor which induces a broad spectrum of cell apoptosis [49, 50], the number of earliest-stage apoptotic cells was significantly increased to 264.3% of the control with no staurosporine ($p < 0.01$); pretreatment with standard GLP-1 reduced the apoptosis to 170% ($p < 0.05$) (Fig 5B). The number of earliest-stage apoptotic cells was 582% of the control upon pretreatment with pMG76e culture, while the number was significantly reduced to 376% ($p < 0.01$) when pretreatment was with pMG76e-exendin-4 culture. When wortmannin was added, apoptotic cell number following pMG76e-exendin-4 treatment was 562%, not significantly different from the pMG76e treatment. These results showed that the exendin-4 secreted by *L. paracasei* L14 decreases staurosporine-induced apoptosis via the PI3K signal pathway.

Delivery of exendin-4 by L14/pMG76e-exendin-4 through Caco-2 cell monolayer

The effect of L14/pMG76e-exendin-4 on cell viability in the Caco-2 monolayer was analyzed by the trypan blue method. As shown in Fig 6A, cell viability was 100, 99 and 98% at 2, 4 and 6 h compared to no treatment, respectively. Differences in Caco-2 cell viability in the absence vs. presence of L14/pMG76e-exendin-4 were not significant ($p > 0.05$).

TEER was monitored to explore the integrity of the Caco-2 cell monolayer throughout the experimental period. As shown in Fig 6B, during the transport studies, the TEER values were nearly stable and there was no statistical difference between the wells in the presence or absence of L14/pMG76e-exendin-4, indicating that the integrity of the cell monolayer was not compromised.

Fig 6C shows the absorption of exendin-4 through the Caco-2 monolayer when delivered by the transformed strain L14/pMG76e-exendin-4 or standard exendin-4 solution (control). During the first 3 h, the exendin-4 transport rates through the cell monolayer was 0.18 ± 0.002 and 0.15 ± 0.013 pMol cm^{-2} h^{-1} for L14/pMG76e-exendin-4 and the standard exendin-4, respectively. However, from 3–6 h, the transport rate of L14/pMG76e-exendin-4-delivered exendin-4 reached 2.5 ± 0.45 pMol cm^{-2} h^{-1} (Fig 6D), which was 34 times that of the exendin-4 control, indicating that the living *L. paracasei* delivery system has a much higher efficiency for exendin-4 absorption and transportation. This high delivery efficiency is probably due to the bacteria's strong adherence to the monolayer surface leading to an increased concentration of exendin-4 close to the epithelial cell monolayer [31, 51].

Discussion

Exendin-4, a long-acting agonist of the GLP-1 receptor, shares many antidiabetic actions with GLP-1, and has become an important therapeutic drug for diabetes. However, subcutaneous injection is still the common administration route for such peptide drugs.
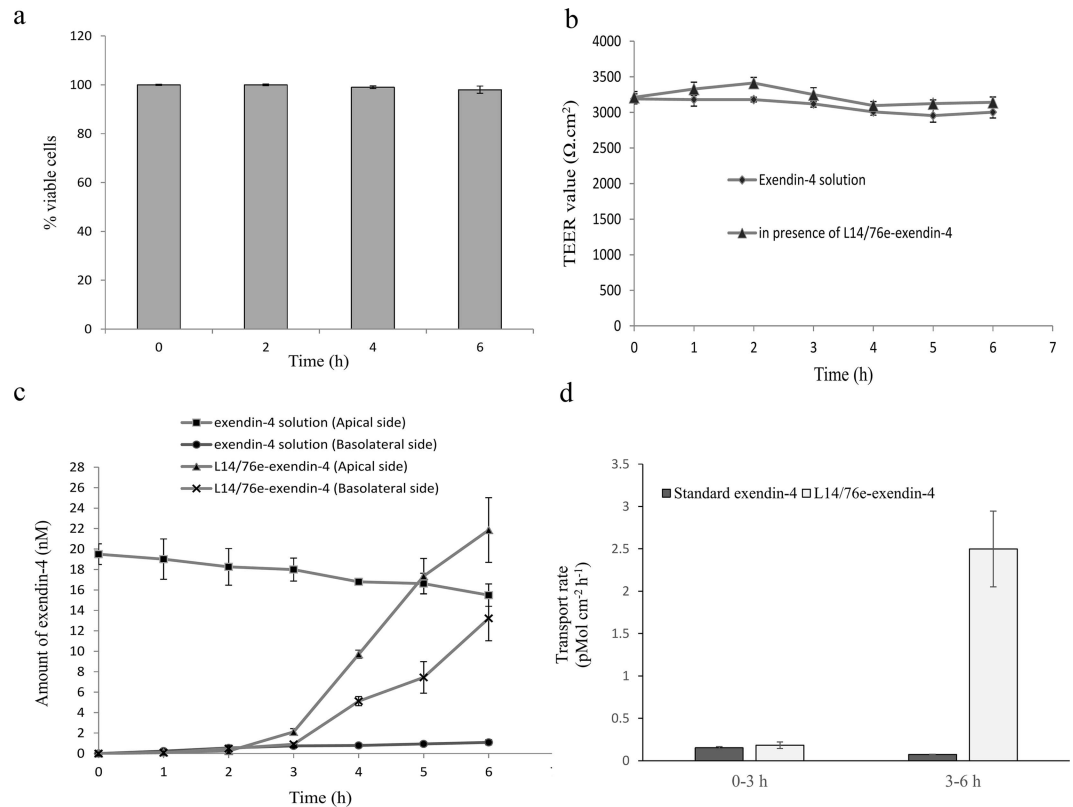
Fig 6. Transport of exendin-4 through Caco-2 cell monolayers. (a) Caco2 cell viability when co-cultured with strain L14/pMG76e-exendin-4. (b) TEER values of the Caco-2 cell monolayer in the presence of L14/pMG76e-exendin-4 or standard exendin-4 solution (control) during the exendin-4 transport study (means ± SD, n = 3). L14/pMG76e-exendin-4 suspension in s-DMEM or standard exendin-4 in s-DMEM was added to the apical side of all wells and incubated for 6 h. (c) Transport of exendin-4 across the Caco-2 monolayers (means ± SD, n = 3). Samples from both the apical and basolateral side of the transwell were taken for the assay of exendin-4 concentration by ELISA (means ± SD, n = 3). (d) Normalized transport rate of exendin-4 through Caco-2 monolayers (means ± SD, n = 3).

In this study, we developed a novel oral delivery system for exendin-4 by using an engineered *Lactobacillus* strain as the carrier. Lactobacilli, as the major inhabitants of the human intestinal tract, can reach and colonize the intestine and play an important role in maintaining a healthy microbiota, as well as contributing to many beneficial functions for human health [29]. These properties make lactobacilli better candidates as live oral vectors for mucosal delivery of prophylactic and therapeutic molecules than *Lactococcus lactis*. We developed recombinant *L. paracasei* as an oral vector for the live delivery of exendin-4 *in situ*, taking advantage of its probiotic colonization of the intestinal tract.

To guarantee an engineered strain of *L. paracasei* L14 with considerable exendin-4 secretion, the Usp45 signal peptide and LEISSTCDA were concatenated as the signal and leading peptides of recombinant exendin-4 [52] (Fig 1). Western blot confirmed expression of the exendin-4 proprotein in both supernatant and cells of the bacteria (Fig 2A); cleavage of the recombinant signal peptide, yielding mature exendin-4 with the expected molecular mass of 5.1 kDa, was obtained from the proteoforms of exendin-4 under the proper pH conditions (Fig 2B), due to the putative influence of medium pH on the processing of the secreted recombinant protein. When grown in MRS medium for 12 h, *L. paracasei* L14 acidifies the growth medium to pH 4.0–4.6, whereas when it is grown in buffered RPMI 1640 medium for 12 h, the pH value

is maintained at 6.0–7.4. Previous studies have suggested that culture pH is an important factor in regulating secreted recombinant protein for correct sorting, processing, and maintenance of stability and bioactivity. It was further revealed that an acid environment decreases the stability and bioactivity of secreted proteins [33, 53, 54,]. Thus, we hypothesize that the mature exendin-4 develops in buffered RPMI 1640 medium primarily because its near-neutral pH leads to better protein processing. In addition, studies have reported that lactobacilli reside mainly in the human ileum, jejunum, and colon [55, 56], which have pH values of about 6.0–8.0 [57]. These values are close to that of the buffered RPMI 1640 medium in which the mature exendin-4 developed.

Quantification assay showed secretion of 28.5 nmol/l exendin-4 after 12 h (Fig 2C). When co-cultured with Caco-2 cells, total secretion of exendin-4 reached 70.4 pMol/10^8 cfu within 6 h following a linear increment, and about 51% of this was absorbed and transported by the Caco-2 cell monolayer (Fig 6C). The secreted and processed exendin-4 protein had an insulinotropic effect on the rat pancreatic islet β-cell line INS-1, comparable to the positive control (100 nmol/l GLP-1) (Fig 3). The GLP-1 analog exendin-4 is known to have important antidiabetic effects, mainly through promotion of insulin secretion and insulin synthesis via upregulation of *PDX*-1 gene expression [47, 58], facilitation of β-cell neogenesis and inhibition of β-cell apoptosis [15]. Our results indicated that the secreted exendin-4 retains biological activity for enhancement of β-cell insulin secretion and synthesis, which is related to the PDX-1-dependent pathway (Figs 3 and 4). Increased β-cell proliferation and inhibition of β-cell apoptosis were also observed (Fig 5). As displayed in Fig 5, *L. paracasei* L14 harboring exendin-4 significantly stimulated cell proliferation and decreased cell apoptosis compared to the strain harboring the empty vector pMG76e. In contrast, when the inhibitor wortmannin was added, there was no significant difference in either cell proliferation or apoptosis following treatment with L14/pMG76e-exendin-4 vs. L14/pMG76e. This proves that the biological activity of recombinant exendin-4 is in fact through the GLP-1 receptor pathway, which comprises the PI3K signaling pathway when exendin-4 is delivered by the *L. paracasei* L14 strain. Compared to the blank control (only cell media), *L. paracasei* L14 harboring pMG76e and pMG76e-exendin-4 both inhibited cell growth. This could be due to inhibition by *L. paracasei* L14 itself. Several studies have reported that probiotic lactic acid bacteria have anticancer effects on cancer cells but have no cytotoxicity toward normal cells [59, 60]. Since INS-1 is a rat insulinoma cell line, inhibition due to *L. paracasei*'s anticancer effects is a reasonable assumption. Taken together, our results demonstrate that the engineered strain *L. paracasei* L14/pMG76e-exendin-4 can efficiently express and secrete the recombinant exendin-4, and by *in situ* delivery, transform the target protein into biologically active exendin-4, as a GLP-1 receptor agonist.

The Caco-2 cell line, a widely used intestinal model for predicting *in vivo* intestinal drug absorption [61], was used to model for the transport of secreted exendin-4 *in vitro*. The transport rate of exendin-4 delivered by L14/pMG76e-exendin-4 was 34-fold higher than that of the exendin-4 solution from 3–6 h of the experimental period (Fig 6D). Our results are in accordance with previous studies in which the transport rate of GLP-1 was increased by eight times and that of β-lactamase was doubled by *L. lactis* delivery [35, 51]. The concentration of exendin-4 on the Caco-2 monolayer may be an important reason for the absorption enhancement. Since the *L. paracasei* strain shows strong adhesion to the monolayer, it can secrete exendin-4 directly onto the apical surface of the monolayer and result in a locally high concentration gradient.

This study represents a novel trial for the use of *L. paracasei* as a system for oral delivery of exendin-4 for diabetes treatment. As such, it opens up the possibility of oral exendin-4 delivery using live food-grade microorganisms. Many studies have reported that probiotics can control diabetes by improving insulin resistance and alleviating hyperglycemia [62–64]. *L. paracasei*

L14 was isolated from traditional dairy products, with natural and safe properties, and our previous study also showed that it possesses DPP-IV-inhibitory activity [65] and strong adhesive capacity for intestinal epithelial HT-29 cells, 3.2-fold that of *Lactobacillus rhamnosus* GG. Thus, the recombinant *L. paracasei* L14 can colonize the intestinal mucosa and continuously express and deliver the peptide drug *in situ* for long periods. This is desirable and important for diabetes and other chronic diseases. The constitutive p32 promoter used here can maintain the expression of exendin-4 in a cell density-dependent manner without the aid of any expression inducer, such as nisin, after oral administration.

Although the present study shows that *L. paracasei* can secrete bioactive exendin-4 and significantly increases the transport of exendin-4 through Caco-2 monolayers, this is only an initial step in exploring the possibility of oral exendin-4 delivery by *L. paracasei*. Some issues still need to be addressed before clinical trials, such as the efficacy of *in vivo* delivery, control of the dose of recombinant protein for the desired effect, and potential environmental pollution by the engineered bacteria when eliminated from the body.

Conclusions

The present work demonstrates that, when transformed by a plasmid, probiotic *L. paracasei* can be a live delivery vector for the peptide drug exendin-4. Using lactobacilli as delivery vectors for diabetes treatment opens up the possibility of oral exendin-4 delivery using live food-grade microorganisms. Further investigations into the biological activity of the engineered *Lactobacillus* strain *in vivo* are therefore warranted.

Supporting Information

S1 Fig. The effect of glucose concentration on GLP-1-stimulated insulin secretion from INS-1 cells. Cells were cultured in media with different concentrations of glucose with or without GLP-1 for 2 h, and then insulin concentration was assayed. Medium with 3 mM glucose was used as a control. Data represent the means ± SD of three independent experiments. (TIF)

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References

1. Drucker DJ. Glucagon-like peptides. Diabetes. 1998; 47: 159–169 PMID: 9519708

2. Nauck MA, Wollschlager D, Werner J, Holst JJ, Ørskov C, Creutzfeldt W, et al. Effects of subcutaneous glucagon-like peptide 1 (GLP-1 [7–36 amide]) in patients with NIDDM. Diabetologia. 1996; 39: 1546–1553 PMID: 8960841

3. Willms B, Werner J, Holst JJ, Orskov C, Creutzfeldt W, Nauck MA. Gastric emptying, glucose responses and insulin secretion after a liquid test meal: Effects of exogenous glucagon-like peptide-1 (GLP-1)-(7–36) amide in type 2 (noninsulin-dependent) diabetic patients. J Clin Endocrinol Metab. 1996; 81: 327–332 doi: 10.1210/jcem.81.1.8550773 PMID: 8550773

4. Wang Q, Li L, Xu E, Wong V, Rhodes C, Brubaker PL. Glucagon-like peptide-1 regulates proliferation and apoptosis via activation of protein kinase B in pancreatic INS-1 beta cells. Diabetologia. 2004; 47: 478–487 doi: 10.1007/s00125-004-1327-5 PMID: 14762654

5. Verspohl EJ. Novel therapeutics for type 2 diabetes: Incretin hormone mimetics (glucagon-like peptide-1 receptor agonists) and dipeptidyl peptidase-4 inhibitors. Pharmacol Therapeut. 2009; 124: 113–138

6. Zander M, Madsbad S, Madsen JL, Holst JJ. Effect of 6-week course of glucagon-like peptide 1 on glycaemic control, insulin sensitivity, and beta-cell function in type 2 diabetes: a parallel-group study. Lancet. 2002; 359: 824–830 doi: 10.1016/S0140-6736(02)07952-7 PMID: 11897280

7. Sarkar G, Alattar M, Brown RJ, Quon MJ, Harlan DM, Rother KI. Exenatide treatment for 6 months improves insulin sensitivity in adults with type 1 diabetes. Diabetes Care. 2014; 37: 666–670 doi: 10.2337/dc13-1473 PMID: 24194508

8. Holst JJ, Deacon CF. Inhibition of the activity of dipeptidyl-peptidase IV as a treatment for type 2 diabetes. Diabetes. 1998; 47: 1663–1670 PMID: 9792533

9. Neumiller JJ. Incretin-based therapies. Med Clin North Am. 2015; 99(1): 107–129 doi: 10.1016/j.mcna.2014.08.013 PMID: 25456646

10. Mest HJ, Mentlein R. Dipeptidyl peptidase inhibitors as new drugs for the treatment of type 2 diabetes. Diabetologia. 2005; 48(4): 616–620 doi: 10.1007/s00125-005-1707-5 PMID: 15770466

11. Eng J, Kleinman WA, Singh L, Singh G, Raufman JP. Isolation and characterization of exendin-4, an exendin-3 analogue, from Heloderma suspectum venom. Further evidence for an exendin receptor on dispersed acini from guinea pig pancreas. J Biol Chem. 1992; 267: 7402–7405 PMID: 1313797

12. Edwards CM, Stanley SA, Davis R, Brynes AE, Frost GS, Seal LJ, et al. Exendin-4 reduces fasting and postprandial glucose and decreases energy intake in healthy volunteers. Am J Physiol Endocrinol Metab. 2001; 281: E155–E161 PMID: 11404233

13. Buse JB, Henry RR, Han J, Kim DD, Fineman MS, Baron AD. Effects of exenatide (exendin-4) on glycemic control over 30 weeks in sulfonylurea treated patients with type 2 diabetes. Diabetes Care. 2004; 27: 2628–2635 PMID: 15504997

14. Kendall DM, Riddle MC, Rosenstock J, Zhuang DL, Kim DD, Fineman MS, et al. Effects of exenatide (exendin-4) on glycemic control over 30 weeks in patients with type 2 diabetes mellitus treated with metformin and a sulfonylurea. Diabetes Care. 2005; 28: 1083–1091 PMID: 15855571

15. Xu G, Kaneto H. GLP-1/exendin-4 facilitates β-cell neogenesis in rat and human pancreatic ducts. Diabetes Res Clin Pract. 2006; 73: 107–110 doi: 10.1016/j.diabres.2005.11.007 PMID: 16406191

16. Drucker DJ, Nauck MA. The incretin system: glucagonlike peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. The Lancet. 2006; 368 (9548): 1696–1705

17. Bydureon (exenatide extended-release for injectable suspension) [package insert]. Wilmington, DE: AstraZeneca Pharmaceuticals LP; 2014

18. Shaji J, Patole V. Protein and peptide drug delivery: oral approaches. Indian J Pharm Sci. 2008; 70 (3): 269–277 doi: 10.4103/0250-474X.42967 PMID: 20046732

19. Rekha MA, Sharma CP. Oral delivery of therapeutic protein/peptide for diabetes–Future perspectives. Int J Pharm. 2013; 440: 48–62 doi: 10.1016/j.ijpharm.2012.03.056 PMID: 22503954

20. Bouttefeux O, Beloqui A, Préat V. Delivery of peptides via the oral route: diabetes treatment by peptide-loaded nanoparticles. Curr Pharm Design. 2016; 22: 1161–1176

21. Fonte P, Araujo F, Silva C, Pereira C, Reis S, Santos HA, et al. Polymer-based nanoparticles for oral insulin delivery: revisited approaches. Biotechnol Adv. 2015; 33: 1342–1354 doi: 10.1016/j.biotechadv.2015.02.010 PMID: 25728065

22. Gupta V, Hwang BH, Doshi N, Banerjee A, Anselmo AC, Mitragotri S. Delivery of exenatide and insulin using mucoadhesive intestinal devices. Ann Biomed Eng. 2016; 44(6): 1993–2007 doi: 10.1007/s10439-016-1558-x PMID: 26864536

23. Sánchez-Navarro M, Garcia J, Giralt E, Teixidó M. Using peptides to increase transport across the intestinal barrier. Adv Drug Deliv Rev. 2016. http://dx.doi.org/10.1016/j.addr.2016.04.031

24. Castro PM, Fonte P, Sousa F, Madureira AR, Sarmento B, Pintado ME. Oral films as breakthrough tools for oral delivery of proteins/peptides. J Control Release. 2015; 211: 63–73 doi: 10.1016/j.jconrel. 2015.05.258 PMID: 25979328

25. Lopes M, Simoes S, Veiga F, Seica R, Ribeiro A. Why most oral insulin formulations do not reach clinical trials. Ther Deliv. 2015; 6: 1–15

26. Wyszyńska A, Kobierecka P, Bardowski J, Jagusztyn-Krynicka EK. Lactic acid bacteria—20 years exploring their potential as live vectors for mucosal vaccination. Appl Microbiol Biotechnol. 2015; 99: 2967–2977 doi: 10.1007/s00253-015-6498-0 PMID: 25750046

27. Cano-Garrido O, Seras-Franzoso J, Garcia-Fruitós E. Lactic acid bacteria: reviewing the potential of a promising delivery live vector for biomedical purposes. Microb Cell Fact. 2015; 14: 137 doi: 10.1186/ s12934-015-0313-6 PMID: 26377321

28. Wang W, Gao ZQ, Zhong YG, Pan L. Lactic acid bacteria as mucosal delivery vehicles: a realistic therapeutic option. Appl Microbiol Biotechnol. 2016; 100: 5691–5701 doi: 10.1007/s00253-016-7557-x PMID: 27154346

29. Gareau MG, Sherman PM, Walker WA. Probiotics and the gut microbiota in intestinal health and disease. Nat Rev Gastroenterol Hepatol. 2010; 7: 503–514 doi: 10.1038/nrgastro.2010.117 PMID: 20664519

30. Ouwehand AC, Kirjavainen PV, Gronlund M-M, Isolauri E, Salminen S. Adhesion of probiotic microorganisms to intestinal mucus. Int Dairy J. 1999; 9: 623–630

31. Kaushal G, Trombetta L, Ochs RS, Shao J. Delivery of TEM beta-lactamase by gene-transformed *Lactococcus lactis* subsp. *lactis* through cervical cell monolayer. Int J Pharm. 2006; 313(1–2): 29–35 doi: 10.1016/j.ijpharm.2006.01.013 PMID: 16488095

32. Braat H, Rottiers P, Hommes DW, Huyghebaert N, Remaut E, Remon JP, et al. A phase I trial with transgenic bacteria expressing interleukin-10 in Crohn's disease. Clin Gastroenterol Hepatol. 2006; 4 (6): 754–759 doi: 10.1016/j.cgh.2006.03.028 PMID: 16716759

33. Ng DT, Sarkar CA. Nisin-inducible secretion of a biologically active single-chain insulin analog by *Lactococcus lactis* NZ9000. Biotechnol Bioeng. 2011; 108: 1987–1996 doi: 10.1002/bit.23130 PMID: 21391210

34. Ma Y, Liu J, Hou J, Dong Y, Lu Y, Jin L, et al. Oral administration of recombinant *Lactococcus lactis* expressing HSP65 and tandemly repeated P277 reduces the incidence of type I diabetes in non-obese diabetic mice. PLOS ONE. 2014; 9: e105701 doi: 10.1371/journal.pone.0105701 PMID: 25157497

35. Agarwal P, Khatri P, Billack B, Low W-K, Shao J. Oral delivery of glucagon like peptide-1 by a recombinant *Lactococcus lactis*. Pharm. Res. 2014; 31: 3404–3414 doi: 10.1007/s11095-014-1430-3 PMID: 24928365

36. Robert S, Gysemans C, Takiishi T, Korf H, Spagnuolo I, Sebastiani G, et al. Oral delivery of glutamic acid decarboxylase (GAD)-65 and IL10 by *Lactococcus lactis* reverses diabetes in recent-onset NOD mice. Diabetes. 2014; 63: 2876–2887 doi: 10.2337/db13-1236 PMID: 24677716

37. Wei P, Yang Y, Li T, Ding Q, Sun H. An engineered *Bifidobacterium longum* secreting a bioactive penetratin-Glucagon-like peptide 1 fusion protein enhances Glucagon-like peptide 1 absorption in the intestine. J Microbiol Biotechn. 2015; doi: 10.4014/jmb.1412.12030 PMID: 25674803

38. Duan FF, Liu JH, March JC. Engineered commensal bacteria reprogram intestinal cells into glucose-responsive insulin-secreting cells for the treatment of diabetes. Diabetes. 2015; 64: 1794–1803 doi: 10.2337/db14-0635 PMID: 25626737

39. van de Guchte M, van der Vossen JMBM, Kok J, Venema G. Construction of a lactococcal expression vector: expression of hen egg white lysozyme in *Lactococcus lactis* subsp. *lactis*. Appl Environ Microbiol. 1989; 55: 224–228 PMID: 2495760

40. Schägger H. Tricine-sds-page. Nat Protoc. 2006; 1: 16–22 doi: 10.1038/nprot.2006.4 PMID: 17406207

41. Zhang B, Li AD, Zuo FL, Yu R, Zeng Z, Ma HQ, et al. Recombinant *Lactococcus lactis* NZ9000 secretes a bioactive kisspeptin that inhibits proliferation and migration of human colon carcinoma HT-29 cells. Microb Cell Fact. 2016; 15: 102 doi: 10.1186/s12934-016-0506-7 PMID: 27287327

42. Hohmeier HE, Mulder H, Chen GX, Henkel-Rieger R, Prentki M, Newgard CB. Isolation of INS-1–derived cell lines with robust ATP-sensitive K+ channel-dependent and-independent glucose-stimulated insulin secretion. Diabetes. 2000; 49: 424–430 PMID: 10868964

43. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. Nat Protoc. 2008; 3: 1101–1108 PMID: 18546601

44. Hubatsch I, Ragnarsson EGE, Artursson P. Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. Nat Protoc. 2007; 2: 2111–2119 doi: 10.1038/nprot.2007.303 PMID: 17853866

45. Habener JF, Stoffers DA. A newly discovered role of transcription factors involved in pancreas development and the pathogenesis of diabetes mellitus. Proc Assoc Am Physicians. 1998; 110: 12–21 PMID: 9460079

46. Stoffers DA, Kieffer TJ, Hussain MA, Drucker DJ, Bonner-Weir S, Habener JF, et al. Insulinotropic glucagon-like peptide 1 agonists stimulate expression of homeodomain protein IDX-1 and increase islet size in mouse pancreas. Diabetes. 2000; 49: 741–748 PMID: 10905482

47. Wang X, Zhou J, Doyle ME, Egan JM. Glucagon-like peptide-1 causes pancreatic duodenal homeobox-1 protein translocation from the cytoplasm to the nucleus of pancreatic beta-cells by a cyclic adenosine monophosphate/protein kinase A-dependent mechanism. Endocrinology. 2001; 142: 1820–1827 doi: 10.1210/endo.142.5.8128 PMID: 11316746

48. Thum A, Hupe-Sodmann K, Goke R, Voigt K, Goke B, McGregor GP. Endoproteolysis by isolated membrane peptidases reveal metabolic stability of glucagon-like peptide-1 analogs, exendins-3 and -4. Exp Clin Endocrinol Diabetes. 2002; 110: 113–118 doi: 10.1055/s-2002-29087 PMID: 12012270

49. Belmokhtar CA, Hillion J, Ségal-Bendirdjian E. Staurosporine induces apoptosis through both caspase-dependent and caspase-independent mechanisms. Oncogene. 2001; 20(26): 3354–3362 doi: 10.1038/sj.onc.1204436 PMID: 11423986

50. Thuret G, Chiquet C, Herrag S, Dumollard JM, Boudard D, Bednarz J, et al. Mechanisms of staurosporine induced apoptosis in a human corneal endothelial cell line. Br J Ophthalmol. 2003; 87(3): 346–352 PMID: 12598452

51. Shao J, Kaushal G. Normal flora: living vehicles for non-invasive protein drug delivery. Int J of Pharm. 2004; 286: 117–124

52. Le Loir Y, Nouaille S, Commissaire J, Brétigny L, Gruss A, Langella P. Signal peptide and propeptide optimization for heterologous protein secretion in *Lactococcus lactis*. Appl Environ Microbiol. 2001; 67 (9): 4119–4127 doi: 10.1128/AEM.67.9.4119-4127.2001 PMID: 11526014

53. Schotte L, Steidler L, Vandekerckhove J, Remaut E. Secretion of biologically active murine interleukin-10 by *Lactococcus lactis*. Enzyme Microb Technol. 2000; 27(10): 761–765 PMID: 11118583

54. Khokhlova EV, Efimov AB, Kafarskaia LI, Shkoporov AN. Heterologous expression of secreted biologically active human interleukin-10 in Bifidobacterium breve. Arch Microbiol. 2010; 192: 769–774 doi: 10.1007/s00203-010-0606-4 PMID: 20631991

55. Wells JM, Mercenier A. Mucosal delivery of therapeutic and prophylactic molecules using lactic acid bacteria. Nat Rev Microbiol. 2008; 6(5): 349–362 doi: 10.1038/nrmicro1840 PMID: 18345021

56. Hartstra AV, Bouter KEC, Bäckhed F, Nieuwdorp M. Insights into the role of the microbiome in obesity and type 2 diabetes. Diabetes Care. 2015; 38: 159–165 doi: 10.2337/dc14-0769 PMID: 25538312

57. Charman WN, Porter CJ, Mithani S, Dressman JB. Physiochemical and physiological mechanisms for the effects of food on drug absorption: The role of lipids and pH. J Pharm Sci. 1997; 86(3): 269–282 doi: 10.1021/js960085v PMID: 9050793

58. Chepurny OG, Hussain MA, Holz GG. Exendin-4 as a stimulator of rat insulin I gene promoter activity via bZIP/CRE interactions sensitive to serine/threonine protein kinase inhibitor Ro 31–8220. Endocrinology. 2002; 143: 2303–2313 doi: 10.1210/endo.143.6.8870 PMID: 12021195

59. Hirayama K, Rafter J. The role of lactic acid bacteria in colon cancer prevention: mechanistic considerations. Antonie van Leeuwenhoek. 1999; 76: 391–394 PMID: 10532395

60. Nami Y, Abdullah N, Haghshenas B, Radiah D, Rosli R, Khosroushahi AY. Probiotic potential and biotherapeutic effects of newly isolated vaginal *Lactobacillus acidophilus* 36YL strain on cancer cells. Anaerobe. 2014; 28: 29–36 doi: 10.1016/j.anaerobe.2014.04.012 PMID: 24818631

61. Pinto M, Robine-Leon S, Appay M-D, Kedinger M, Triadou N, Dussaulx E, et al. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. Biol Cell.1983; 47: 323–330

62. Cani PD, Neyrinck AM, Fava F, Knauf C, Burcelin RG, Tuohy KM, et al. Selective increases of bifidobacteria in gut microflora improve high-fat diet- induced diabetes in mice through a mechanism associated with endotoxaemia. Diabetologia. 2007; 50: 2374–2383 doi: 10.1007/s00125-007-0791-0 PMID: 17823788

63. Baboota RK, Bishnoi M, Ambalam P, Kondepudi KK, Sarma SM, Boparai RK, et al. Functional food ingredients for the management of obesity and associated co-morbidities–A review. J Funct Foods. 2013; 5: 997–1012

64. Miyoshi M, Ogawa A, Higurashi S, Kadooka Y. Antiobesity effect of *Lactobacillus gasseri* SBT2055 accompanied by inhibition of pro-inflammatory gene expression in the visceral adipose tissue in diet-

induced obese mice. Eur J Nutr. 2014; 53: 599–606 doi: 10.1007/s00394-013-0568-9 PMID: 23917447

65.   Zeng Z, Luo JY, Zuo FL, Zhang Y, Ma HQ, Chen SW. Screening for potential novel probiotic *Lactobacillus* strains based on high dipeptidyl peptidase IV and α-glucosidase inhibitory activity. J Funct Foods. 2016; 20: 486–495