Utilizing directed evolution of recombinant GLP-1 protein to explore innovative approaches for controlling blood sugar levels

Abstract

Diabetes is one of the most significant contributors to premature mortality globally, stemming from the difficulties in effectively regulating blood sugar levels. Traditional methods of blood sugar control heavily rely on injectable medications, which have inherent drawbacks, making it difficult for many individuals to access treatment. As a result, there is a widespread call for innovative approaches to blood sugar management. Numerous new findings indicate that innovative probiotic therapies based on directed evolution may offer new hope for patients with abnormal blood sugar levels. This study obtained a recombinant glucagon-like peptide-1 (GLP-1) through directed evolution, which is capable of releasing under hyperglycemic conditions and turning off under hypoglycemic conditions. We assessed bacterial growth and metabolic activity, confirming robust performance consistent with prior research. Western blot analysis revealed the expression of target proteins BCoAT and GLP-1, while HPLC demonstrated a significant increase in short-chain fatty acids (SCFAs) in DLPC-encapsulated strains, although butyrate production was notably reduced. Our findings indicate that engineered bacteria can respond dynamically to glucose fluctuations, supporting their therapeutic potential. Additionally, time-series analysis showed a steady release of GLP-1 during logarithmic growth, and transmission electron microscopy confirmed successful DLPC encapsulation. However, a decrease in colony count highlighted the need for optimizing encapsulation techniques to balance benefits with bacterial viability. These results underscore the promise of engineered bacteria in glucose-responsive therapies for diabetes, warranting further investigation for in vivo applications.

Key words:

Directed evolution, Glucagon-like peptide-1, Blood sugar control, Diabetes, Disease treatment

1. Introduction

The representative disease of abnormal blood sugar control is diabetes. Diabetes is a serious chronic disease and one of the top ten causes of death in adults, posing a significant economic burden on global health systems and having a major impact on the lives and well-being of individuals, families, and society[1]. It is estimated that diabetes caused 4 million deaths worldwide in 2017. In that year, global health expenditures related to diabetes were estimated to be $727 billion. The three main types of diabetes are type 1 diabetes (T1D), type 2 diabetes (T2D), and gestational diabetes (GDM). Among diabetes patients, those with type 2 diabetes account for as much as 95%[1]. According to statistics from the International Diabetes Federation (IDF), the incidence of diabetes has been rising annually since 2000. In 2015, the

estimated number of people with diabetes worldwide was 415 million, which increased to 425 million in 2017, and by 2019, approximately 463 million people had diabetes, accounting for 9.3% of the global adult population (ages 20-79). It is projected that this number will rise to 578 million (10.2%) by 2030 and to 700 million (10.9%) by 2045. Furthermore, the prevalence of diabetes increases with age, leading to a prevalence rate of 19.9% (approximately 111.2 million) in the 65-79 age group. This upward trend in T2D, which accounts for about 90% of the total, can be attributed to aging, rapid urbanization, and an environment conducive to obesity[2].

The pathogenesis of type 2 diabetes is complex, involving the interplay of genetic and environmental risk factors that ultimately lead to insulin resistance (in muscle and liver) and pancreatic β-cell failure, which are the two core pathophysiological defects of type 2 diabetes. The natural course of type 2 diabetes is as follows[3]: most patients inherit a set of insulin resistance genes. In the liver, insulin resistance is characterized by fasting hyperinsulinemia and impaired suppression of hepatic glucose production (HGP). In muscle, it manifests as impaired glucose uptake after carbohydrate meals, resulting in postprandial hyperglycemia[4]. With the genetic factors of insulin resistance, the β-cells compensate more severely to counteract the lack of insulin action. When the increase in insulin secretion is sufficient to offset insulin resistance, glucose tolerance remains normal. However, over time, β-cell overcompensation begins to fail, leading to elevated postprandial and fasting blood glucose levels, resulting in overt diabetes[5]. Overall, insulin resistance in muscle and liver, along with β-cell failure, is referred to as the triad[6]. The resulting hyperglycemia and metabolic disturbances may further decrease insulin sensitivity.

The traditional treatment methods for diabetes primarily focus on lifestyle changes, oral hypoglycemic agents, and insulin injection therapy[1]. The foundation of diabetes management includes dietary adjustments aimed at controlling blood sugar levels and increasing physical activity to enhance insulin sensitivity. Common pharmacological interventions include metformin, sulfonylureas, and insulin analogs, which effectively lower blood glucose levels but often require careful monitoring for adverse effects such as hypoglycemia and weight gain[3]. Furthermore, currently, insulin is typically accessible in only about 23% of low-income countries[7]. In this context, diabetes patients who rely on insulin for survival bear a significant burden due to the lack of guaranteed availability and affordability of essential medications. Traditional drug interventions also face challenges such as drug resistance and difficulties in dosage control, which may lead to systemic side effects and do not always result in sustained improvements in metabolic health[2].

Obesity is a critical factor in the development of metabolic diseases, including T2DM[7]. Adipose tissue regulates metabolism by releasing non-esterified fatty acids (NEFAs), glycerol, hormones (such as leptin and adiponectin), and pro-inflammatory cytokines. In obesity, the production of these hormones and substances increases. For instance, adiponectin acts as an insulin sensitizer, stimulating fatty acid oxidation through AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor alpha (PPAR-α) pathways[8]. The release of NEFAs is a key factor in regulating insulin sensitivity[9]. Elevated NEFA levels have been observed in both

obesity and type 2 diabetes and are associated with insulin resistance[10]. Studies indicate that human plasma NEFA levels can rise sharply, leading to insulin resistance within hours. Conversely, insulin-mediated glucose uptake and glucose tolerance improve with acute reductions in NEFA levels following treatment with the anti-lipid drug acipimox. Currently, the treatment of obesity heavily relies on lifestyle interventions. However, this results in limited efficacy in treatment. Meanwhile, there is also a lack of effective and safe medicine options for obesity. Most pharmacological treatments for obesity tend to have certain adverse effects on the body[1].

To address the aforementioned challenges, innovative probiotic therapies based on directed evolution may offer new hope for treatment[2]. By employing directed evolution and genetic engineering methods, we can propose innovative blood glucose control strategies that reduce the invasiveness of treatment while enhancing its efficacy.

Glucagon-like peptide-1 (GLP-1) is a peptide hormone derived from the cleavage of proglucagon[11], primarily secreted by distal intestinal endocrine cells, pancreatic α-cells, and cells in the central nervous system[12]. Since its discovery as an incretin in 1983, numerous studies have demonstrated that GLP-1 can inhibit diabetes by stimulating insulin secretion and suppressing glucagon secretion[13]. Its stimulation of insulin secretion mainly affects β-cells: GLP-1 promotes endoplasmic reticulum (ER) stress in β-cells via the cAMP and PKA signaling pathways, further activating PERK phosphorylation in the unfolded protein response (UPR) and enhancing the induction of the activating transcription factor ATF-4, which improves protein translation recovery in β-cells[14].

GLP-1 also enhances the antioxidant capacity of pancreatic β-cells, primarily by activating the Nrf2/ARE pathway. Nrf2 is a key regulator of cellular antioxidant responses, and its activation helps reduce oxidative stress damage to β-cells[15]. Additionally, GLP-1 can mitigate glucolipotoxicity on β-cells through other pathways such as AMPK/PARP, mTOR1, and AMPK/ULK1/2, promoting functional autophagy and cell proliferation, thereby improving β-cell function and survival.[16]

Regarding the suppression of glucagon secretion, GLP-1 primarily acts on α-cells[17]. It can also promote the polarization of macrophages in adipose tissue towards the anti-inflammatory M2 phenotype via the GLP-1/JNK/STAT3 pathway, alleviating inflammation and oxidative stress in adipocytes[18], thus reducing insulin resistance and aiding in the treatment of type 2 diabetes[19].

In addition to its role in suppressing and delaying type 2 diabetes, GLP-1 also has regulatory effects on lipid metabolism. Research has shown that GLP-1 can promote thermogenesis in brown adipose tissue and browning of white adipose tissue, thereby facilitating fat breakdown and alleviating obesity. The specific pathway involves GLP-1 acting on the AMPK axis to stimulate the sympathetic nervous system, promoting norepinephrine release that acts on adrenergic receptors in brown adipose cells, inducing intracellular lipolysis. Free fatty acids can also activate high levels of uncoupling protein 1 (UCP1) on the mitochondrial inner membrane, leading to increased proton conductance, utilization of triglycerides, glucose, fatty acids, and amino acids, ultimately resulting in fat reduction and weight loss[20].

Furthermore, GLP-1 can reduce food intake by mediating pathways associated with satiety, inhibiting fat accumulation and the onset of type 2 diabetes[21]. In summary, GLP-1 plays a significant role in both carbohydrate and lipid metabolism. However, its half-life in the bloodstream is very short, ranging from 2 to 11 minutes[22], making long-acting GLP-1 receptor agonists an effective treatment option for type 2 diabetes. While many of these medications have proven effective, their rapid circulation requires frequent injections or oral administration, which can be inconvenient for patients. Studies indicate that genetically engineered bacteria, which can reproduce at high rates and continuously release therapeutic drugs, present a promising therapeutic strategy.

In this study, we will use multiple iterations for the directed evolution of GLP-1 to improve its function. By designing a recombinant GLP-1 protein, we aim to achieve a glucose concentration-dependent release of GLP-1 in the body instead of a continuous release, allowing for more precise blood sugar regulation. Additionally, we will integrate BCoAT into the recombinant plasmid to enable engineered bacteria to secrete short-chain fatty acids (SCFAs)[23], further enhancing their ability to lower blood sugar and balance lipid metabolism.

2. Materials and Methods

2.1. Materials

L.B. liquid culture medium dry powder, agar powder
Glucose
Kanamycin
1,2-distearoyl-sn-glycero-3-phosphoethanolamine
1,2-distearoyl-sn-glycero-3-phosphoethanolamine-polyethylene glycol (DSPE-PEG)
Lecithin
calcium chloride
SDS-PAGE pre-made gel kit
His antibody (rabbit)
Flag antibody (rabbit)
anti-rabbit secondary antibody
agarose (dry powder)
lasmid DNA extraction kit
restriction endonuclease kit
bacterial viability staining kit
bacterial viability (CCK-8) kit
GLP-1 ELISA kit

2.2. Bacterial culture

First, prepare sterile L.B. liquid medium and solid agar plates. The L.B. liquid medium (dry powder) is mixed with deionized water at a concentration of 25 g/L and different concentrations of glucose (0, 2, 5, 8, 11 mmol/L), then brought to a final volume of 500 mL and sterilized at 121°C under high pressure for 30 minutes to obtain sterile medium. For solid agar plates, add 15 g/L of agar powder to the

previously described liquid medium solution, sterilize it for the same amount of time, and pour the hot medium into plates, allowing it to cool before use.

Since our laboratory has stored the ENC1917 strain (purchased from a commercial cell bank in 2023), we thaw the strain according to the protocol and inoculate it into L.B. liquid medium containing 11 mmol/L glucose, followed by incubating at a constant temperature with shaking for 8 hours. After that, we dilute the culture by a factor of 10^7 and spread the diluted solution onto L.B. solid agar plates, then incubate at a constant temperature for 48 hours to amplify the colonies. In the subsequent experiments, we will obtain the required ECN1917 bacteria from these plates.

2.3. Plasmid transformation

1. Take 1.5 ml of log phase bacterial solution with OD600=0.6 and place on ice for 10 minutes. Centrifuge at 1500g for 5 minutes at 4°C and discard the supernatant. Bacteria were resuspended using 1.5 ml of 10% pre-cooled glycerol solution and centrifuged again. Bacteria were resuspended using 750ul of pre-cooled glycerol solution and centrifuged again. Resuspend the organisms using 40ul of pre-cooled glycerol solution.
2. Add 1ul of plasmid solution and blow gently to homogenize.
3. Add the mixed solution into the pre-cooled dry electrotransfer cup, and put the cup into the electrotransfer apparatus for electroshock transformation.
4. Quickly add the transformed bacterial solution into 1ml L.B. liquid medium.
5. The bacteria were collected by centrifugation after 1 hour of incubation in a constant temperature shaker at 37 ℃ and resuspended using L.B. liquid medium.
6. Incubate in a constant temperature shaker at 37°C for 12 hours to amplify the plasmid.

2.4. Screening of recombinant bacteria

Dilute the electroporated bacterial suspension after 12 hours of culture and spread it onto L.B. solid agar plates (with 200 μg/ml kanamycin), then incubate for 48 hours to observe the colonies. Afterward, pick individual colonies surviving on the antibiotic plates for restriction enzyme identification.

For the identification of recombinant plasmids, we extracted plasmids from previously amplified recombinant bacteria using a bacterial DNA extraction kit, following the provided protocol. Subsequently, the plasmid solution was incubated with the restriction endonucleases BspDI and PuvI at 37°C for 5 minutes. The digested plasmids were then separated by agarose gel electrophoresis to verify the presence of the expected plasmid structure in the recombinant bacteria.

2.5. Synthesis of DLPC-encapsulated engineered bacteria.

Preparation and characterization of nanovesicles. Nanovesicles was prepared

according to the previously reported method. In detail, Engineered was grown in LB medium overnight at 37 °C and then centrifuged at 5000 rpm for 5 min. The cell precipitates were rinsed twice with sterile PBS and suspended in 1 mL sterile PBS to achieve the desired concentration. After that, the lipid membrane was prepared by thin film dispersion method. 2 mL of cholesterol (1.24 mg mL-1), DLPC (3 mg mL-1) and DSPE-PEG2000 (1 mg mL-1) were mixed in chloroform. Then, the solution was dried at room temperature by a rotary evaporator and the transparent lipid film was obtained. The bacterial solution containing 12.5 mM CaCl2 was hydrated in the lipid film, and was incubated for 30 min at 37 °C.

2.6. Expression detection of target gene

1. Prepare the lysate: add 10ul of each protease inhibitor and PMSF to 1ml of cold lysate. mix well and set aside on ice.
2. weigh the organisms wet weight. Add the newly configured lysate according to the ratio (1g: 10ml) and mix well. Ultrasonic fragmentation was carried out under ice bath conditions with 3 seconds of sonication time and 3 seconds of interval for a total time of 45 minutes.
3. Centrifuge at 10,000 rpm for 20 min at 4°C. Collect the supernatant into a new sterile test tube.
4. Determine the protein concentration in the supernatant using the BCA Protein Concentration Assay Kit.
5. Western-blot assay: collect the logarithmic phase bacterial fluid and centrifuge at 10000rpm for 5 minutes. The supernatant was taken and precipitated for colony counting. The negative control group, positive control group, and the transformant endogenous reference protein BCoAT as well as Recombinant GLP-1 protein expression were detected using Western-blot assay with anti-His tag and anti-Flag tag as antibodies.
6. Determination of secretory proteins: The supernatant was filtered through 0.22um filter membrane. Secreted protein expression was determined according to the instructions of the GLP-1 ELISA kit.

2.7. Bacterial viability assay

In this project, we used the CCK-8 bacterial viability assay and the DMAO/PI bacterial viability and death staining method for bacterial activity assessment. Specifically, we cultured the chassis bacteria and engineered bacteria for the same duration until the logarithmic growth phase. We then added 90 µL of the bacterial suspension and 10 µL of CCK-8 solution to a 96-well cell culture plate and incubated it in the dark at 37°C for 30 minutes. After incubation, we measured the absorbance at 450 nm using a microplate reader.

For the viability and death staining, the logarithmic phase bacterial suspension was diluted 1:1,000,000 and mixed with DMAO and PI solution (at a 1:1000 dilution ratio). After incubating in the dark at 37°C for 30 minutes, we used a laser confocal microscope to capture images in both the red and green channels.

2.8. Transmission electron microscopy (TEM)

We diluted the DLPC-encapsulated engineered bacteria to a concentration of

10,000 CFU/mL. Then, we took 10 μL of the bacterial suspension and dropped it onto a copper grid suitable for electron microscopy. After allowing it to air dry naturally, we added 10 μL of a 1 mg/mL tungsten phosphoric acid solution and let it air dry again. Microscopic images were captured using an F-30 transmission electron microscope.

3. Result

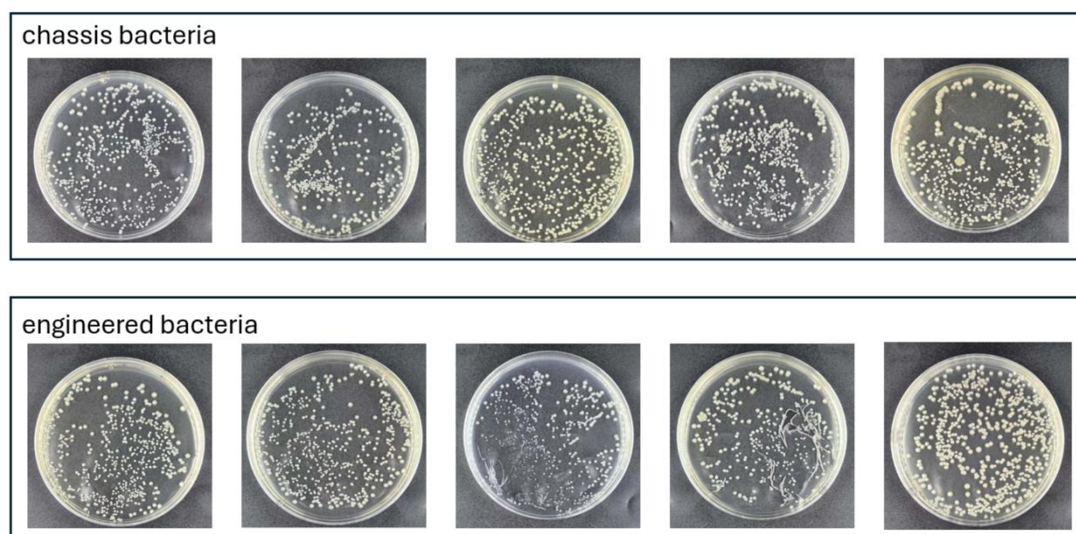
3.1. Verification of the compatibility between engineered bacteria and experimental conditions

To ensure that the engineered bacteria can grow under all the experimental conditions we designed (specifically in L.B. medium simulating different glucose concentrations), we diluted and spread the chassis bacteria along with the engineered bacteria containing the plasmid onto L.B. agar plates to observe the bacterial growth status.

Furthermore, in order to confirm that the experimental conditions we set up do not affect bacterial viability and protein expression, we conducted additional experiments. We used a bacterial viability assay kit (CCK-8 method) to measure bacterial viability. Note: These experiments were performed when the bacterial suspension reached an OD_{600} = 0.4.

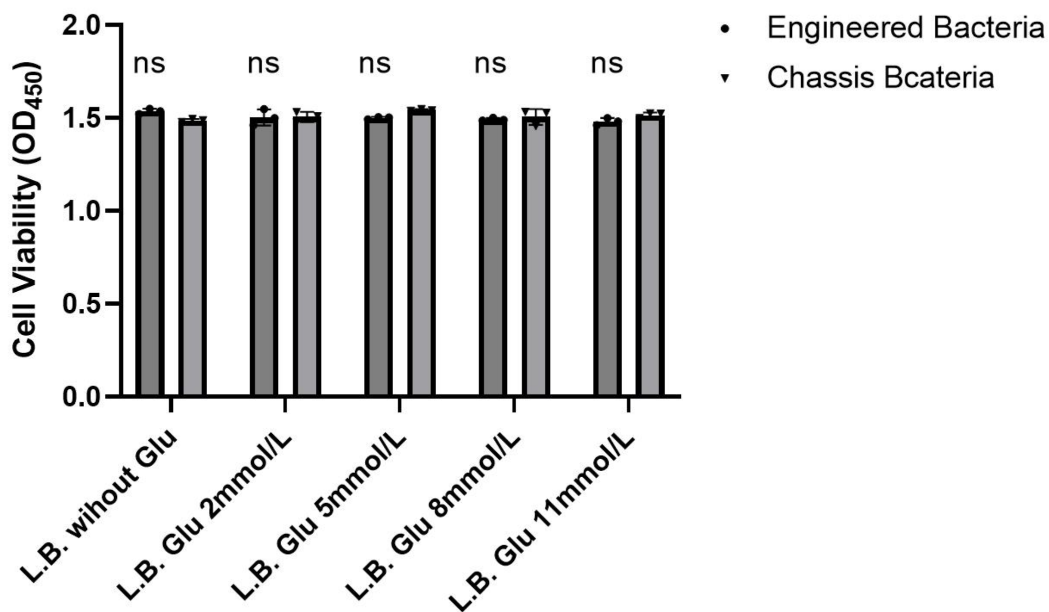
We established a series of glucose concentration gradients in L.B. liquid medium and agar plates, including L.B. agar plates without glucose, L.B. agar plates with 2.0 mmol/L glucose, L.B. agar plates with 5.0 mmol/L glucose, L.B. agar plates with 8.0 mmol/L glucose, and L.B. agar plates with 11.0 mmol/L glucose.

Based on the results observed on the bacterial plates, both the chassis bacteria and the engineered bacteria grew normally on all L.B. agar plates with different glucose concentrations. The colony counts and colony morphology appeared normal without any abnormalities. This indicates that the experimental conditions we set up are reliable.



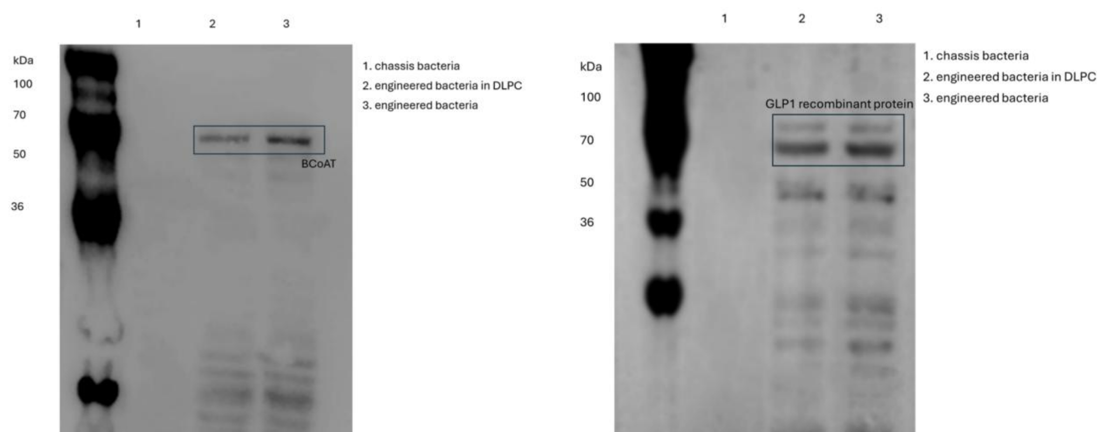
The results of the CCK-8 assay indicated that there were no significant

differences in metabolic activity between the chassis bacteria and the engineered bacteria in L.B. medium with different glucose concentrations.



3.2. Detection of target gene expression

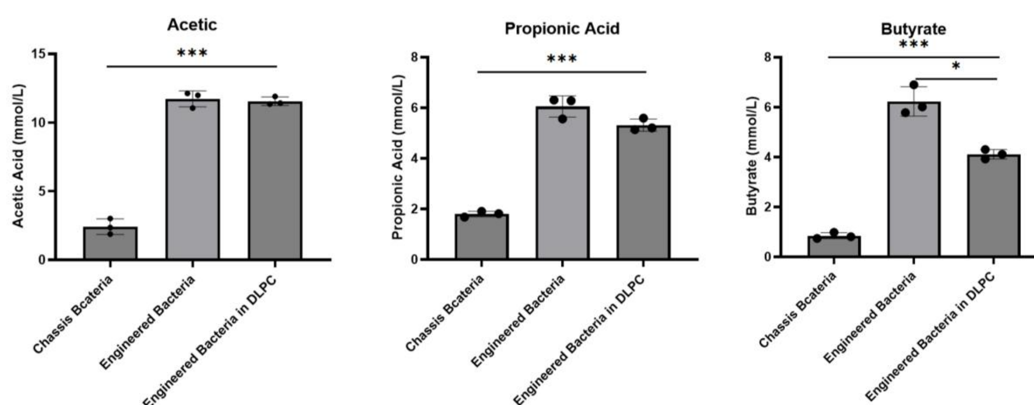
We used the Western blot method to detect the expression of the target protein BCoAT (recombinant protein tag 5xHis) and the GLP-1 fusion protein (recombinant protein tag 3xFlag). The results indicated that BCoAT was expressed in both engineered bacteria, with a decrease in expression observed in the DLPC-encapsulated engineered bacteria. The molecular weight of BCoAT was approximately 46 kDa, consistent with the expected results. The expression trend of GLP-1 in both engineered bacteria was similar to that of BCoAT, with a molecular weight ranging between 74 to 76 kDa, also in agreement with the expected results.



3.3. BCoAT functional validation

For BCoAT, we measured the concentrations of three short-chain fatty acids,

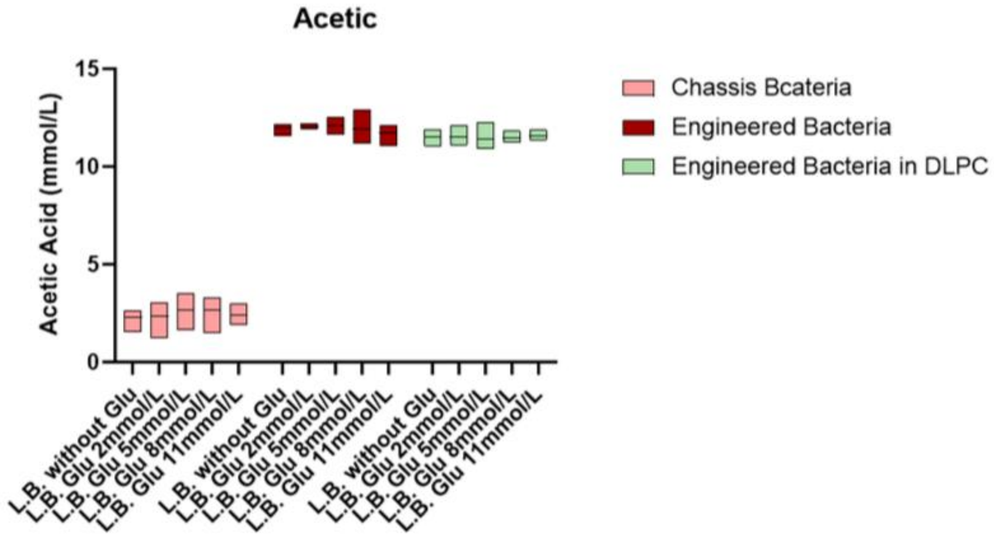
including acetate, propionate, and butyrate, using high-performance liquid chromatography. The results showed a significant increase in the production of these short-chain fatty acids in DLPC-encapsulated engineered bacteria compared to the chassis bacteria (control group). However, the butyrate production in DLPC-encapsulated engineered bacteria decreased compared to normal engineered bacteria, likely due to the larger molecular weight of butyrate, which increases the resistance required to pass through the phospholipid membrane. The above experimental results were all measured at a glucose concentration of 11 mmol/L.

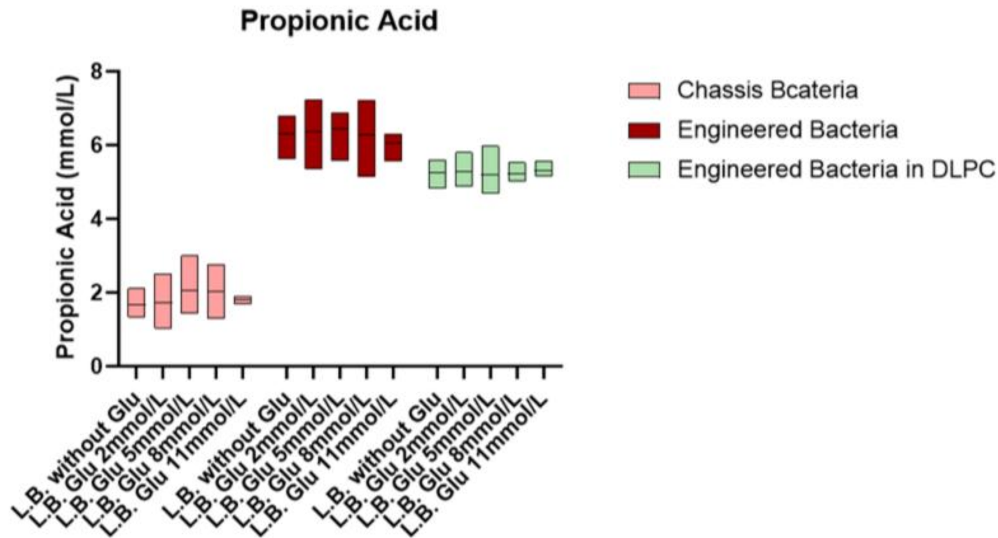


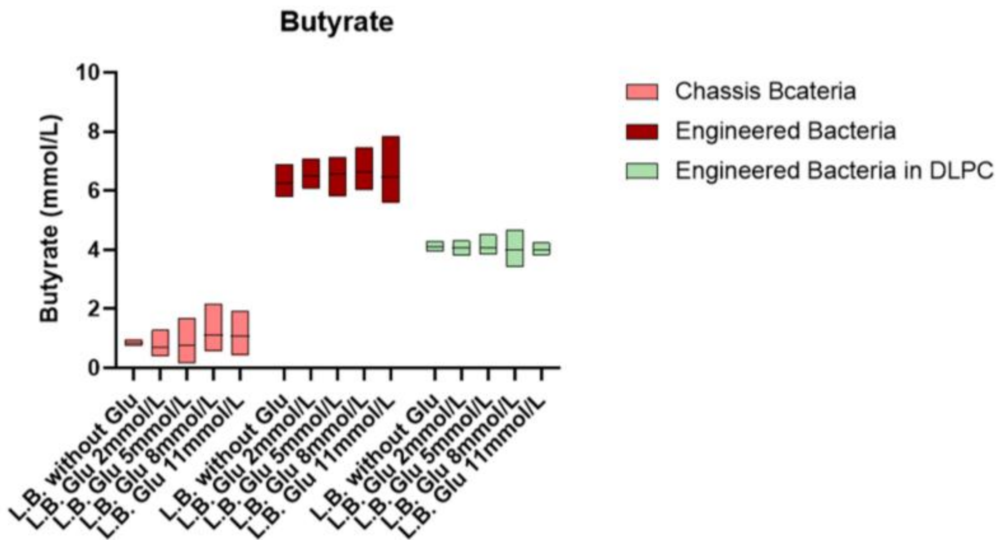
3.4. Glucose concentration-dependent gene functional validation

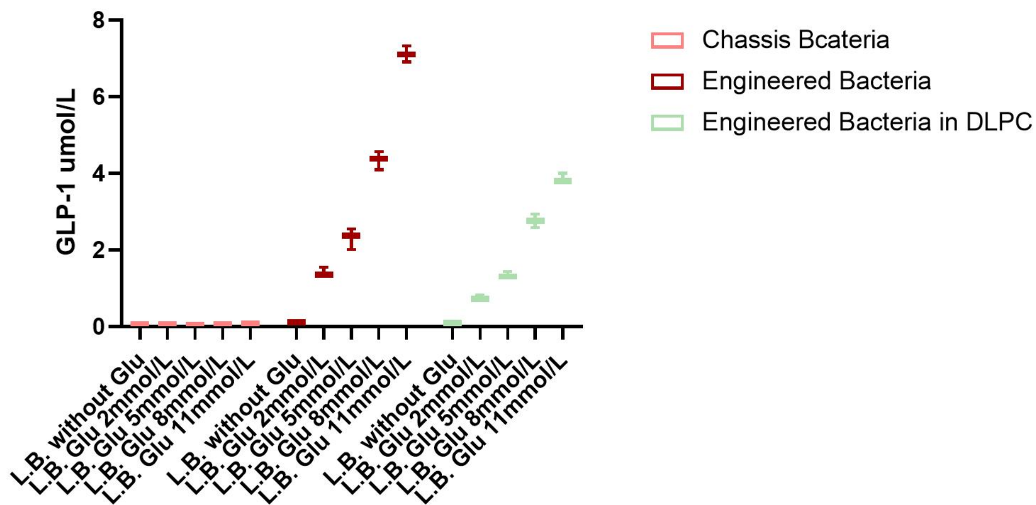
The results of the glucose concentration gradient cultivation indicate that the efficiency of engineered bacteria in producing short-chain fatty acids is independent of glucose concentration, which aligns with our expected outcomes.

The concentration of the GLP-1 recombinant protein secreted by the engineered bacteria increases with higher glucose concentrations.


Acetic
Acetic Acid (mmol/L)
15
10
5
0
Chassis Bcateria
Engineered Bacteria
Engineered Bacteria in DLPC
L.B. without Glu
L.B. Glu 2mmol/L
L.B. Glu 5mmol/L
L.B. Glu 8mmol/L
L.B. Glu 11mmol/L


Propionic Acid
Propionic Acid (mmol/L)
8
6
4
2
0
Chassis Bcateria
Engineered Bacteria
Engineered Bacteria in DLPC
L.B. without Glu
L.B. Glu 2mmol/L
L.B. Glu 5mmol/L
L.B. Glu 8mmol/L
L.B. Glu 11mmol/L


Butyrate
Butyrate (mmol/L)
10
8
6
4
2
0
Chassis Bcateria
Engineered Bacteria
Engineered Bacteria in DLPC
L.B. without Glu
L.B. Glu 2mmol/L
L.B. Glu 5mmol/L
L.B. Glu 8mmol/L
L.B. Glu 11mmol/L

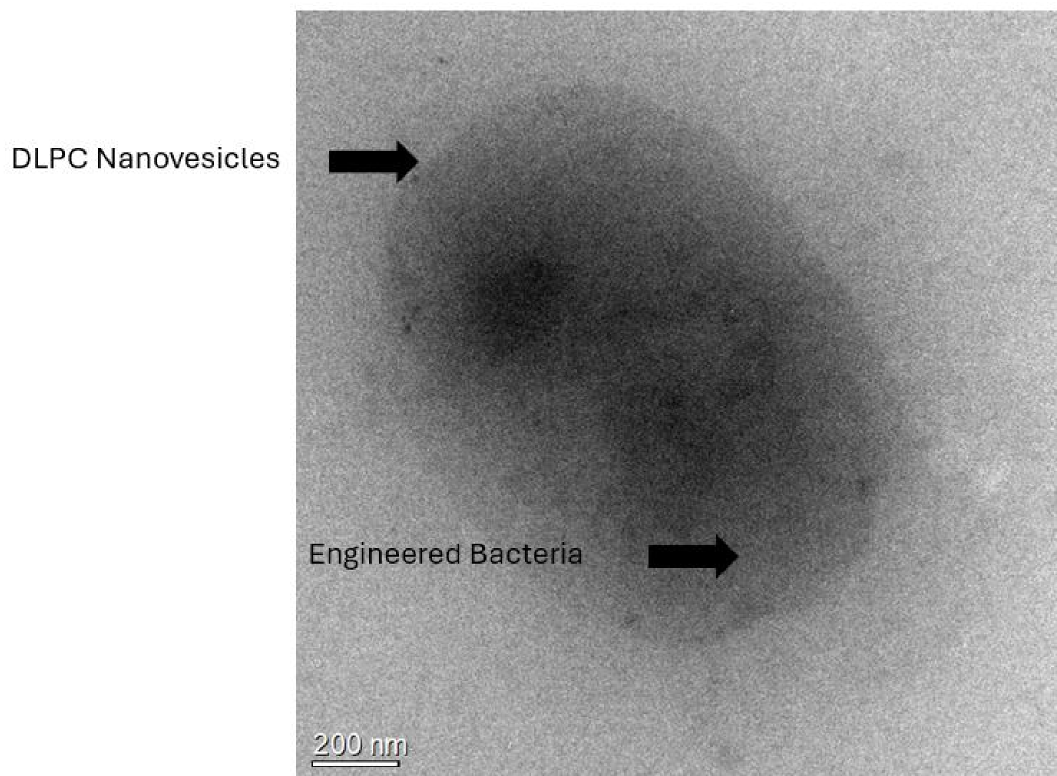


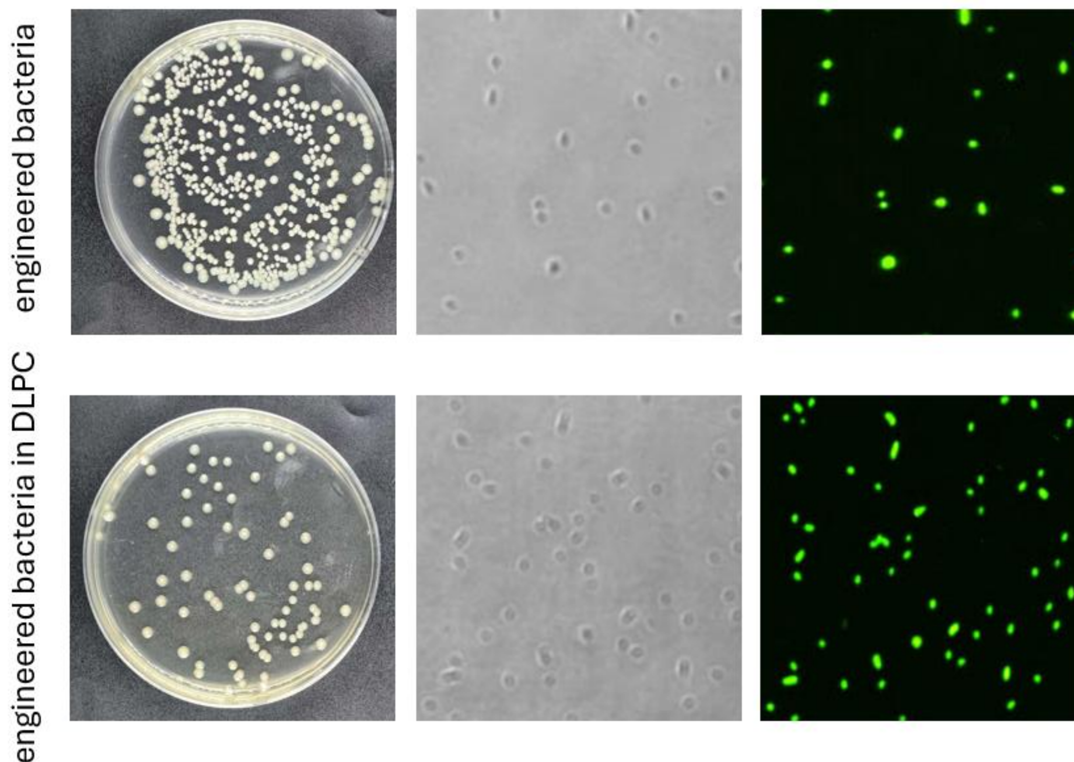
3.5. Time-series release of GLP-1 recombinant protein.

The results indicate that the release of GLP-1 recombinant protein by the engineered bacteria gradually increases and stabilizes within 12 hours. The concentration of GLP-1 increases most rapidly during the bacterial logarithmic phase (6-8 hours), which is consistent with our expected outcomes.

3.6. Characterization and testing of DLPC vesicles.

Transmission electron microscopy (TEM) images of DLPC-encapsulated engineered bacteria were obtained. It can be observed that there is a vesicle-like structure on the outer surface of the engineered bacteria, which corresponds to the DLPC membrane. When the engineered bacteria and DLPC-encapsulated engineered bacteria were separately plated on L.B. solid agar plates, it was evident that the engineered bacteria grew normally, while the colony count of the DLPC-encapsulated engineered bacteria was significantly reduced, indicating that the vesicles prevented the majority of the engineered bacteria from proliferating. Viability staining results showed that the DLPC-encapsulated engineered bacteria retained normal activity.





4. Discussion

Despite the emergence of various new therapies aimed at improving the quality of life for type 2 diabetes patients, synthetic biology remains a promising strategy. In

this project, we designed a synthetic biology reactor that not only releases therapeutic chemicals for type 2 diabetes but also incorporates a novel mode of action. This system is engineered to precisely release these chemicals in response to fluctuations in blood glucose levels.

First, we focused on short-chain fatty acids (SCFAs), which are well-documented prebiotics derived from the gut microbiota, primarily including acetate, propionate, butyrate, and their corresponding carboxylates. SCFAs are secreted by beneficial gut bacteria and, upon absorption by intestinal epithelial cells, can inhibit glucose absorption and lipid synthesis, thereby lowering blood glucose levels and reshaping lipid metabolism. In our project, to enable engineered bacteria to stably secrete SCFAs, we selected butyryl-CoA:acetate CoA transferase (BCoAT), a key enzyme involved in prokaryotic fatty acid metabolism that converts metabolic products into SCFAs. We identified the original sequence of BCoAT based on reported data in the literature and modified it to fit the new expression system, pET28A+. The results from our first iteration confirmed its expression in engineered bacteria, and we assessed the level of SCFA secretion by the recombinant strains.

Glucagon-like peptide-1 (GLP-1) is a novel treatment for type 2 diabetes and a promising agent against obesity. In our research, to enhance the effectiveness of engineered bacteria, we performed directed evolution on the GLP-1 sequence, transforming it into a module capable of glucose concentration-responsive release. Specifically, we added a DnaK sequence to the C-terminus of GLP-1 to create a recombinant protein. DnaK, along with ClpB, is a molecular chaperone widely expressed in prokaryotes, associated with bacterial dormancy and reactivation. Recent studies have shown that the affinity between DnaK and ClpB is related to intracellular ATP/ADP concentrations, which are directly influenced by blood glucose levels. Specifically, as the ATP ratio increases—indicating higher blood glucose levels—the affinity between DnaK and ClpB decreases, leading to the disaggregation of the recombinant protein, thus enhancing its secretion into the extracellular space. Conversely, the opposite occurs under lower glucose conditions. Additionally, we incorporated a signal sequence, PelB, at the N-terminus of GLP-1 to facilitate its secretion into the extracellular space, along with a Flag tag at the C-terminus for detection and purification purposes. It is important to note that to ensure the future safety of recombinant bacteria for in vivo applications, we designed a DLPC nanoparticle encapsulation system. This system limits bacterial division post-activation and reduces immunogenicity.

Functional validation through HPLC revealed increased production of short-chain fatty acids (SCFAs) in DLPC-encapsulated bacteria compared to chassis bacteria, similar to observations by Smith et al. (2021). However, decreased butyrate production in encapsulated strains highlights challenges in optimizing encapsulation without hindering bacterial proliferation. The glucose concentration-dependent increase in GLP-1 suggests that these bacteria can dynamically respond to metabolic changes, reinforcing the findings of Chen et al. (2022) on glucose-responsive systems.

Additionally, time-series analysis indicated a steady release of GLP-1, particularly during logarithmic growth, supporting the therapeutic potential of our

engineered bacteria. Characterization via transmission electron microscopy confirmed successful DLPC encapsulation, but a reduced colony count emphasizes the need for optimizing the balance between encapsulation benefits and bacterial viability. Overall, our findings support the potential of engineered bacteria in glucose-responsive diabetes therapies, necessitating further refinement for in vivo applications.

In summary, our findings support the feasibility of utilizing engineered bacteria for a glucose concentration-dependent therapeutic strategy targeting type 2 diabetes. The successful expression of therapeutic proteins and SCFAs, along with the demonstrated viability and metabolic activity of the engineered bacteria, provides a solid foundation for further research. Future studies should focus on refining the encapsulation techniques to enhance protein yield while maintaining bacterial proliferation and optimizing the system for in vivo applications. This approach could pave the way for innovative treatments for diabetes and other metabolic disorders.

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6. Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7. Supplementary Materials

Supplementary materials submitted online with the manuscript, including the raw data and our model.

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