Synthetic and Systems Biotechnology 6 (2021) 272–282

Contents lists available at ScienceDirect

Synthetic and Systems Biotechnology

journal homepage: www.keaipublishing.com/en/journals/synthetic-and-systems-biotechnology

Antidiabetic effect of an engineered bacterium *Lactobacillus plantarum*-pMG36e -GLP-1 in monkey model

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ARTICLE INFO

Keywords:
L. plantarum-pMG36e-GLP-1
T2DM
Gut microbiome
Faecal metabolomics

ABSTRACT

Glucagon-like peptide-1 (GLP-1) reduces postprandial hyperglycaemia, but its short half-life inhibits clinical application. The aim of the current study was to evaluate the treatment efforts of an engineered strain, *Lactobacillus plantarum*-pMG36e-GLP-1 (*L. plantarum*-pMG36e-GLP-1), that continuously expresses GLP-1 in spontaneous type 2 diabetes mellitus (T2DM) monkeys. After 7 weeks of oral supplementation with *L. plantarum*-pMG36e-GLP-1, the fasting blood glucose (FPG) of monkeys was significantly ($p < 0.05$) reduced to a normal level and only a small amount of weight was lost. The results of metagenomic sequencing showed that *L. plantarum*-pMG36e-GLP-1 caused a substantial ($p < 0.05$) reduction in the intestinal pathogen *Prevotella* and marked enhancement of butyrate-producing *Alistipes* genera. According to the functional analysis using Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways, 19 metabolism-related pathways were significantly enriched in T2DM monkeys after treatment with *L. plantarum*-pMG36e-GLP-1. LC-MS faecal metabolomics analysis found 41 significant differential metabolites (11 higher and 30 lower) in monkeys after treatment pathways linked to the metabolism of cofactors and vitamins were the most relevant. The present study suggests that *L. plantarum*-pMG36e-GLP-1 had an impact on the gut microbial composition and faecal metabolomic profile in spontaneous T2DM monkeys and may be a novel candidate for diabetes treatment.

1. Introduction

Diabetes mellitus (DM) is a frequently-occurring disease endangering people's health, which can lead to a lack of insulin, insulin resistance, and impaired biological function [1]. Because of the significant morbidity caused by complications and mortality, DM is a major public health threat around the world, including in China [2,3]. The disease can result in multiple health problems and complications, such as heart disease, eye problems, impairment of kidney function, nerve damage etc. [4]. DM manifests as two types; type 2 DM (T2DM) accounts for about 85–90% of all cases.

Up to now, there have been many different treatment options that can be effective in T2DM, such as movement treatment, reasonable nutritional therapy and drug treatments. The medications used for the treatment of T2DM include sulfonylurea, biguanide, nateglinide, glucosidase inhibitors and thiazolodinediones [5]. Although these traditional oral antidiabetic agents exert a glucose-lowering effect through improving insulin action and raising the efficiency of insulin secretion, there are also certain side effects, such as gastrointestinal intolerance, leading to impaired liver function and weight gain or worse. Therefore, the development of new anti-diabetic drugs is important. Glucagon-like peptide-1 (GLP-1) is a peptide that can lower blood glucose in many ways and has become a hotspot of research, as GLP-1 analogues and agonists of the GLP-1 receptor have received intense focus [6,7].

GLP-1 is secreted by the intestinal L-cells and can decrease post-meal blood sugar [8]. The mechanism of its hypoglycaemic effects mainly include enhancing the biosynthesis and secretion of insulin, and it also interacts with glucagon as part of its glucose lowering effect, in a sense

Peer review under responsibility of KeAi Communications Co., Ltd.
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https://doi.org/10.1016/j.synbio.2021.09.009
Received 5 July 2021; Received in revised form 12 August 2021; Accepted 13 September 2021

beyond the insulin effect [9–12]. By promoting the secretion of insulin, GLP-1 analogues and dipeptidyl peptidase-4 (DPP-IV) inhibitors have been applied as anti-diabetic drugs [13–15]. As a kind of GLP-1 analogue, Victoza helps the pancreas make more insulin after eating a meal, the short biologic half-life caused by DPP IV-regulated degradation limits GLP-1 as an effective diabetes drug [16].

As a multifactorial disease, diabetes requires more than one treatment of multiple risk factors [17]. The intestinal microbiota is a complicated microbial ecosystem, and keeping the intestinal flora in balance plays an important role in staying healthy [18]. In recent years, the intestinal microbiota has been considered a major component of the human internal environment and is one of the primary environmental agents that determines the severity of diabetes. Animal experiments and population-based studies have shown that there is an intimate relationship between the intestinal microbiota and diabetes [19,20]. Germ-free mice develop type 1 diabetes (T1D), indicating that intestinal microbes contribute to modifying T1D predisposition [21]. Comparing a T2D group to subjects with normal glucose tolerance in another experiment, four *Lactobacillus* species positively correlated with fasting blood glucose and HbA1c (glycosylated haemoglobin) were found to be enriched, while there was negative correlation between five reduced *Clostridium* species and fasting glucose, HbA1c and insulin [22]. Moreover, the microbiota has been reported to autonomously mediate blood glucose [23].

In view of GLP-1's short half-life, our group has obtained an engineered strain *Lactococcus lactis* MG1363-pMG36e-GLP-1 in which GLP-1 is persistently expressed, which showed good treatment results in diet-induced obese mice and in a mouse model of neuropsychiatric disease [24–27]. Since the *Lactococcus lactis* is not a residential bacterium of the human intestine and has poor probiotic characteristics, we constructed a similar engineered strain, *L. plantarum*-pMG36e-GLP-1. *L. plantarum* is a member of the human intestinal microflora and has many physiological functions [28]. In the current study, we administered three spontaneous T2DM rhesus monkeys with the engineered strain *L. plantarum*-pMG36e-GLP-1 to evaluate its potential as a diabetes drug by analysing its effects on the gut microflora and faecal metabolism.

2. Materials and methods

2.1. Animals and administration

Three spontaneous T2DM rhesus monkeys, aged from 13 to 17 years with 1–3 years' course of diabetes, were selected for this study. The selection criteria for the monkeys were according to the report [29] that the fasting plasma glucose (FPG) of the animal should be no more than 5.6 mmol/L [30]. Monkeys were singly raised in large cages with enough space for activities at 22–26 °C and 50–55% humidity under a 12 h light/dark cycle. Besides nutritious food and water, fruit or vegetables were supplied daily, providing 10^9 CFU of the engineered strain *L. plantarum*-pMG36e-GLP-1 that we constructed. The study lasted for 7 weeks. The monkey housing and all experimental protocols used were conducted with the approval of the Committee on the Ethics of Animal Experiments of Nanchang University.

2.2. Body weight measurement and determination of fasting plasma glucose (FPG)

During the two-month study, the body weight and FPG of the three monkeys were measured weekly. The FPG level was determined at 7:00AM (before treatment and each week after start of study) using a Gold AQ Blood Glucose Monitoring System (Sonicare, Changsha, China).

2.3. Microbiota analysis

For the microbiota analysis, faecal samples from the three rhesus monkeys were collected before (C) and after (PC) the administration of *L. plantarum*-pMG36e-GLP-1 for 8 weeks. A genomic DNA kit (Qiagen, Cat # 51804) was used to extract the bacterial genomic DNA, and 1.2% agarose gel electrophoresis and a NanoDrop 2000 UV–vis instrument (Thermo Scientific, Wilmington, DE, USA) were applied for DNA quantification and purity analysis. The 16S rRNA gene region of each sample were amplified and sequenced on an Illumina NovaSeq 6000 platform (GenBank accession No. PRJNA 643924). Libraries were sequenced and paired-end sequencing reads were obtained on the Illumina NovaSeq 6000 platform. Metagenomic sequencing was performed using the shotgun sequencing method. Using FLASH (version1.2.8), the paired-end sequencing reads were merged and divided into the above sequences according to 97% sequence similarity.

2.4. Untargeted faecal metabolomics analysis

The faecal samples collected before (C) and after (PC) the administration of *L. plantarum*-pMG36e-GLP-1 for 8 weeks were mixed with sodium azide and stored at −80 °C. Then, 100 mg (±1%) of each sample was thawed at 4 °C. The extraction process of the metabolites from faeces was performed by adding 600 μL of methanol (−20 °C) containing 2-chlorophenylalanine (4 ppm) and vigorously vortexing for 30 s, and grinding with 100 mg glass beads for 90 s at 60 Hz in high-throughput tissue grinder, followed by sonication for 5 min. Then, 300 μL of the supernatant was carefully transferred for further analysis after centrifugation (12,000 rpm, 4 °C for 10 min). 20 μL of each extract was combined for each test as a quality control (QC) samples. 0.22 μm membranes were applied for the filtration of samples before LC-MS analysis.

The LC-MS experiment was performed on a Thermo Ultimate 3000 system coupled with the Thermo Q Exactive Focus. The LC conditions were set as follows: ACQUITY UPLC® HSS T3 (150 × 2.1 mm, 1.8 μm, Waters) with the temperature of column at 40 °C and the autosampler at 8 °C. Separation was carried out with the following gradient: 2% B/D over 0–1 min, 2%–50% B/D over 1–9 min, 50%–98% B/D over 9–12 min, 98% B/D over 12–13.5 min, 98%–2% B/D over 13.5–14 min, and 14–20 min holding at 2% D in positive mode while 14–17 min at 2% B in negative mode at a flow velocity of 0.25 mL/min, where C is water containing 0.1% formic acid and D is acetonitrile containing 0.1% formic acid in positive mode, while A is 5 mM ammonium formate in water and B is acetonitrile in negative mode. The volume of sample injected was 2 μL. The mass spectrometric experiment was executed using the Thermo Q Exactive Focus mass spectrometer assembled with a positive/negative ion electrospray ionisation (ESI) source. The temperature of the capillary was set to 325 °C and the data were gathered within the range of 81–1000 *m/z* at a mass resolution of 70,000. The prepared QC sample was injected every sixth sample to assess system stability.

The raw data from LC-MS were analysed using R (v3.3.2) to collect the data matrix including retention time (RT), *m/z* values and intensity of peaks. Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were used for distinguishing the overall differences in metabolic profiles in monkeys before and after treatment. Based on OPLS-DA analysis, metabolites with variable importance in the project (VIP) higher than 1 were selected for significance testing. Based on the Kyoto Encyclopaedia of Genes and Genomes (KEGG) and the Human Metabolome Database (HMDB), the ID number of the metabolites were obtained. The metabolites were annotated based on the KEGG database to obtain the general pathway functions of these metabolites. The increased and decreased levels of differentially expressed metabolites were analysed to further explore the impact.

2.5. Statistical data analysis

Statistical analysis was performed with GraphPad Prism software, Version 7.0 (USA). The unpaired *t*-test was applied to analyse significant mean differences between the data of monkeys before and after treatment.

Data are expressed as mean ± standard deviation (X ± SD). A P value below 0.05 was considered a significant difference.

3. Results

3.1. Effect of 7 weeks of L. plantarum-pMG36e-GLP-1 on body weight and FPG

Before the start of the study, the body weight of all the monkeys was about 10 kg and the FPG level was above 5.6 mmol/L. We continually measured the body weight and the FPG level weekly during the treatment with *L. plantarum*-pMG36e-GLP-1. During the study, no change was found in the consumption of food and water. After being treated for 7 weeks, the weight of these monkeys all slightly lower to 9.9 kg, but the FPG level of all the monkeys dropped from 7.33 mmol/L back to 4 mmol/L, which was below the normal level of 5.6 mmol/L (Table 1). The mean values with standard deviation of weekly weight and FPG level during the study are depicted in Fig. 1, and the FPG levels dropped significantly from the second week after treatment. The results reveal that *L. plantarum*-pMG36e-GLP-1 had a marked function on blood sugar reduction and a slight effect on body weight.

3.2. Response of gut bacterial structure to L. plantarum-pMG36e-GLP-1 in T2DM monkeys

To investigate the effect of the engineered *L. plantarum*-pMG36e-GLP-1 on the gut microbiota of T2DM monkeys, we assessed the gut microbiota in faecal samples from monkeys before or after treatment. As is shown in Fig. 2a, the top four phyla in the faecal flora of the monkeys were *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Spirochaetes*. Combined, these phyla accounted for 90.79% and 89.65% of the total sequencing number in the two samples, respectively. *L. plantarum*-pMG36e-GLP-1 treatment increased the relative abundance of *Firmicutes* from 0.52 ± 0.06 to 0.62 ± 0.03 and decreased that of *Bacteroidetes* from 0.32 ± 0.08 to 0.21 ± 0.07 after 7 weeks. At the genus level, administration of *L. plantarum*-pMG36e-GLP-1 increased the abundance of *Clostridium* (8.88% vs. 10.29%) and *Eubacterium* (1.71% vs. 4.07%) that were related to the enhancement of intestinal functions (Fig. 2b). To distinguish the specific bacterial taxa, microbiota in faeces samples of T2DM monkeys before and after *L. plantarum*-pMG36e-GLP-1 treatment were compared by linear discriminant analysis effect size (LEfSe) analysis. There were 65 discriminatory genera with an LDA score higher than 2.5 (Fig. 2c). Among these discriminatory genera, *Prevotella*, *Prevotellaceae*, *Bacteroidetes*, *Bacteroidales*, and *Bacteroidia* were significantly more abundant in the faeces of T2DM monkeys before treatment, whereas *Rikenellaceae*, *Alistipes*, *Alistipes_sp_CAG_435*, *Lactobacillus* and *Lactobacillaceae* were enriched in monkeys after treatment. A cladogram showing the different abundances in the faecal microbiota indicated that the phylogenetic distributions differed significantly between the microflora of T2DM monkeys before and after *L. plantarum*-pMG36e-GLP-1 treatment (Fig. 2d). The results show that the recombinant strain *L. plantarum*-pMG36e-GLP-1 had a considerable impact on the faecal microbiota composition in T2DM monkeys. Finally, the amount of some probiotic microbes and pathogens associated with T2DM were analysed. As shown in Fig. 2, administering *L. plantarum*-pMG36e-GLP-1 enriched *Firmicutes* and *Eubaterium* and significantly enhanced the abundance of *Alistipes* ($P < 0.05$), whereas it reduced the number of pathogenic *Bacteroidetes*, *Bacteroides*, and markedly reduced *Prevotella* ($P < 0.05$).

Table 1
Effect of *L. plantarum*-pMG36e-GLP-1 on body weight and FPG.

	Before initiating the study	After the treatment for 8 weeks
Body weight (kg)	10.27 ± 0.21	9.90 ± 0.32
FPG level (mmol/L)	7.33 ± 0.26	4.00 ± 0.29 *

Significant difference compared with samples before the treatment:$p < 0.05$.

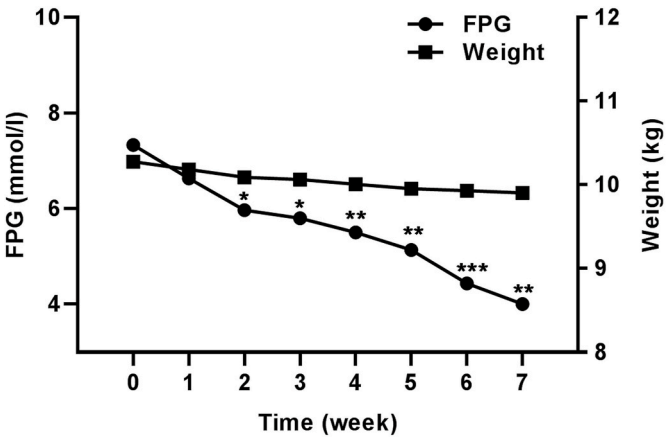

Fig. 1. The fasting blood glucose level (mmol/l) and body weight (kg) of monkeys were assessed weekly during the study. The FPG and weight data are expressed as the mean ± SD, n = 3/group; *P < 0.05, **P < 0.01.

LEfSe was used to study the KEGG pathways with obviously altered abundances in T2DM monkeys subjected to the administration of *L. plantarum*-pMG36e-GLP-1 (Fig. 3). On the basis of the LDA score higher than 2 and P value higher than 0.05, 19 KEGG pathways (including endocrine system, nucleotide excision repair, base excision repair, RNA polymerase and others) were significantly enriched in T2DM monkeys after treatment, and 24 KEGG pathways (including the metabolism of cofactors and vitamins, fructose and mannose metabolism, the metabolism of terpenoids and polyketides, porphyrin and chlorophyll metabolism, and others) were significantly higher in T2DM monkeys before treatment. The results show that *L. plantarum*-pMG36e-GLP-1 affected multiple functional pathways.

3.3. Alterations in the faecal metabolic profile of T2DM monkeys

This investigation adopted LC-MS to analyse faecal samples from monkeys before and after administrating *L. plantarum*-pMG36e-GLP-1. The peak intensity chromatograms of week 0 (C) and week 7 (PC) samples indicated differences both in positive and negative ion modes (Fig. 4). For example, as shown in Fig. 4a, the peaks from 8 min to 9 min in negative ion mode showed a much higher intensity in C samples than that in PC samples. This means that the observation was sufficient to survey for the therapeutic effect of *L. plantarum*-pMG36e-GLP-1.

As is shown in Fig. 4c, the metabolic data of T2DM monkeys before and after treatment were differentiated in the principle component analysis (PCA). All the points representing the composition in the faecal samples of treated T2DM monkeys (PC) were separated from those of monkeys before treatment (C), which means that *L. plantarum*-pMG36e-GLP-1 showed the trends of a curative effect. The data matrix was further analysed by OPLS-DA. As depicted in Fig. 4d, the OPLS-DA score plots clearly separated between the C and PC samples. Based on the parameters that [R^2X (cum) = 0.532, R^2Y (cum) = 1, Q^2(cum) = 0.859] of the OPLS-DA models, it indicated good predictability and reliability of the models, and the abundance of various metabolites in T2DM monkeys before and after administrating *L. plantarum*-pMG36e-GLP-1 was significantly different.

Potential metabolic biomarkers were selected with a VIP value higher than 1.0 in the OPLS-DA and p value below 0.05 (Table 2). There were 41 potential metabolic biomarkers identified in T2DM monkeys before and after treatment. As shown in Fig. 4e, the significantly different abundances of these 41 metabolites were visualised in a heat map. Among these metabolites, 11 were significantly higher and 30 were obviously lower in monkeys after treatment. Based on comparing the peak area and the FC value, the changes in biomarkers after administrating *L. plantarum*-pMG36e-GLP-1 are shown in column configuration (Fig. 5).

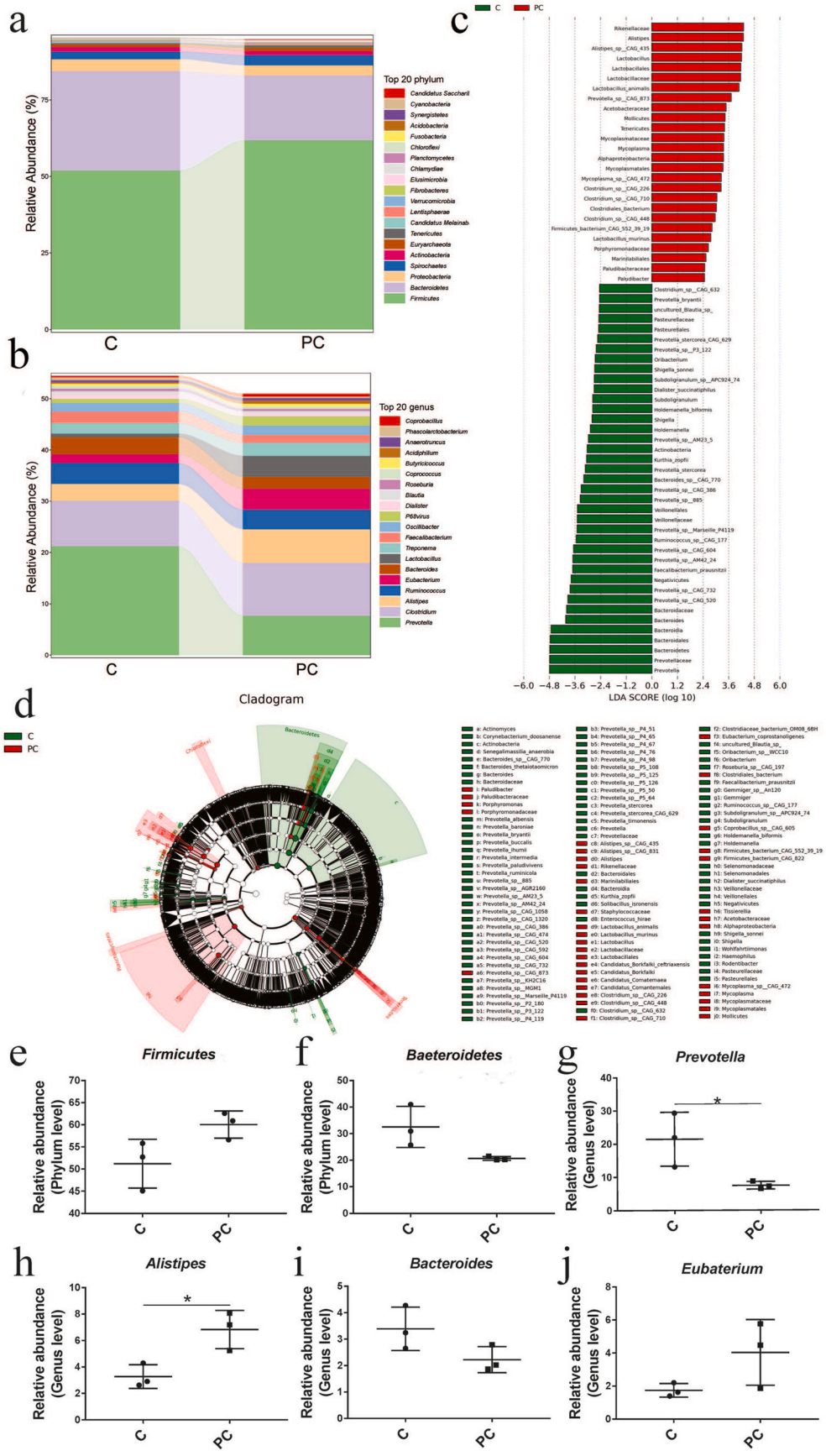

Fig. 2. The *L. plantarum*-pMG36e-GLP-1 affected the intestinal microbiota in T2DM monkeys. (a) The relative abundances of the top 20 phyla; (b) The relative abundances of the top 20 genus; (c) LEfSe analysis between T2DM monkeys before and after treatment (LDA score >2.5); (d) Cladogram showing the phylogenetic distribution of the microbiota of T2DM monkeys before and after treatment ; The relative abundance of *Firmicutes* (e), *Bacteroidetes* (f), *Prevotella* (g) *Alistipes* (h), *Bacteroides* (i) and *Eubaterium* (j) in faeces of T2DM monkeys before and after treatment.

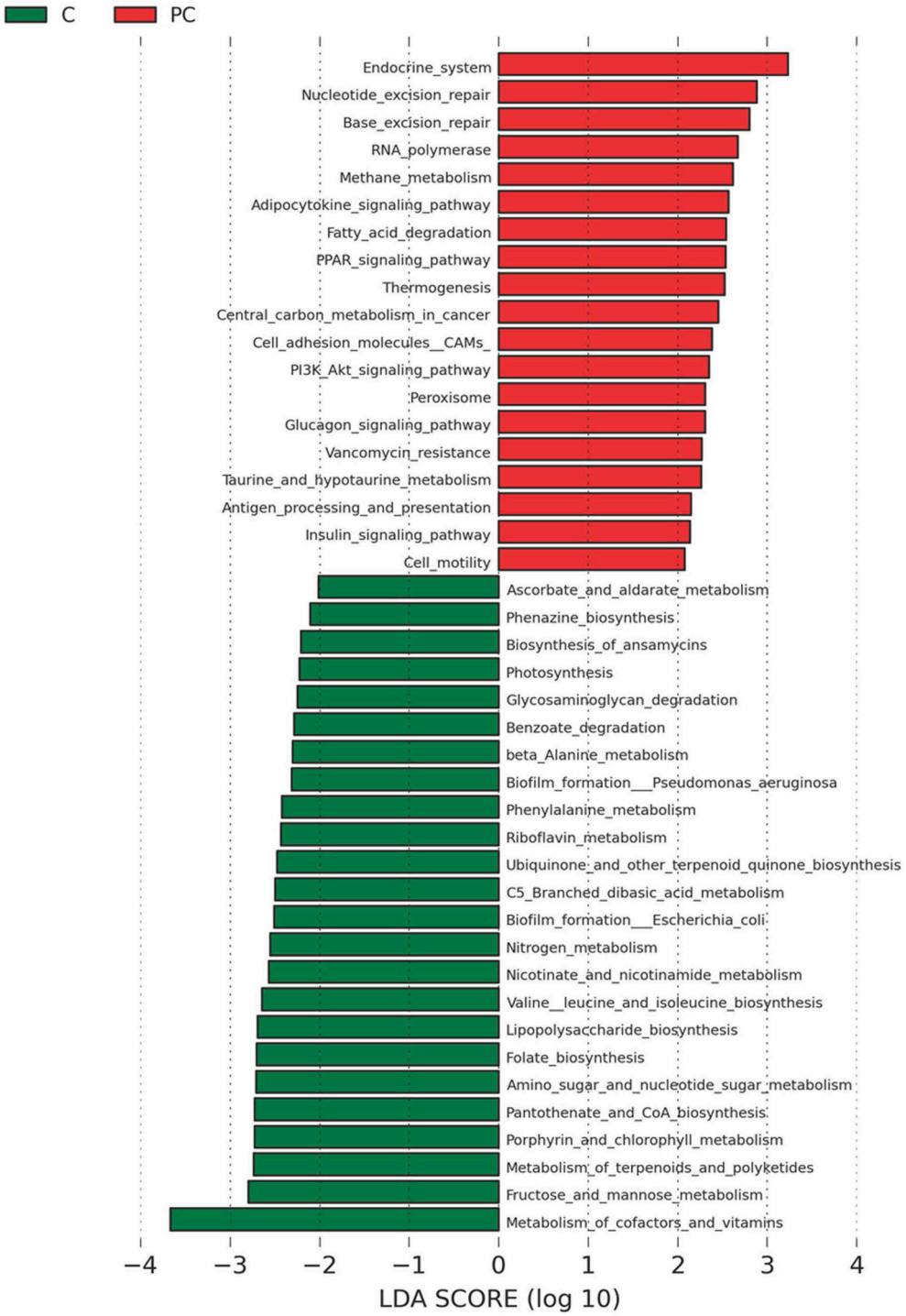

Fig. 3. LEfSe analysis based on the KEGG pathways of the T2DM monkeys before and after treatment (LDA score >2.0, P < 0.05).

In the current study, MetaboAnalyst was applied for correlational analyses between altered metabolites and biochemical pathways. The results show that *L. plantarum*-pMG36e-GLP-1 induced changes in many metabolites that were taking part in many biochemical pathways (Fig. 6 and Table 3). The pathways linked to the metabolism of cofactors and vitamins, such as vitamin B6 metabolism (p = 0.005, impact 0.16) or pantothenate and CoA biosynthesis (p = 0.003, impact 0.08), seemed to be more important. Yet, it is worth noting the three important major forms of metabolites, including amino acids (6), lipids (2), and carbohydrates (3). Additionally, other pathways such as caffeine metabolism (p = 0.00094, impact 0.043) and beta-alanine metabolism (p = 0.003, impact 0.066) were also listed.

4. Discussion

The current study is one of the first to comprehensively evaluate the hypoglycaemic effects of an engineered bacterium, *L. plantarum*-pMG36e-GLP-1, on spontaneous T2DM monkeys. The faecal microbiome of T2DM monkeys before and after the administration of the engineered bacterium was characterised by integrated metagenomic sequencing, and the

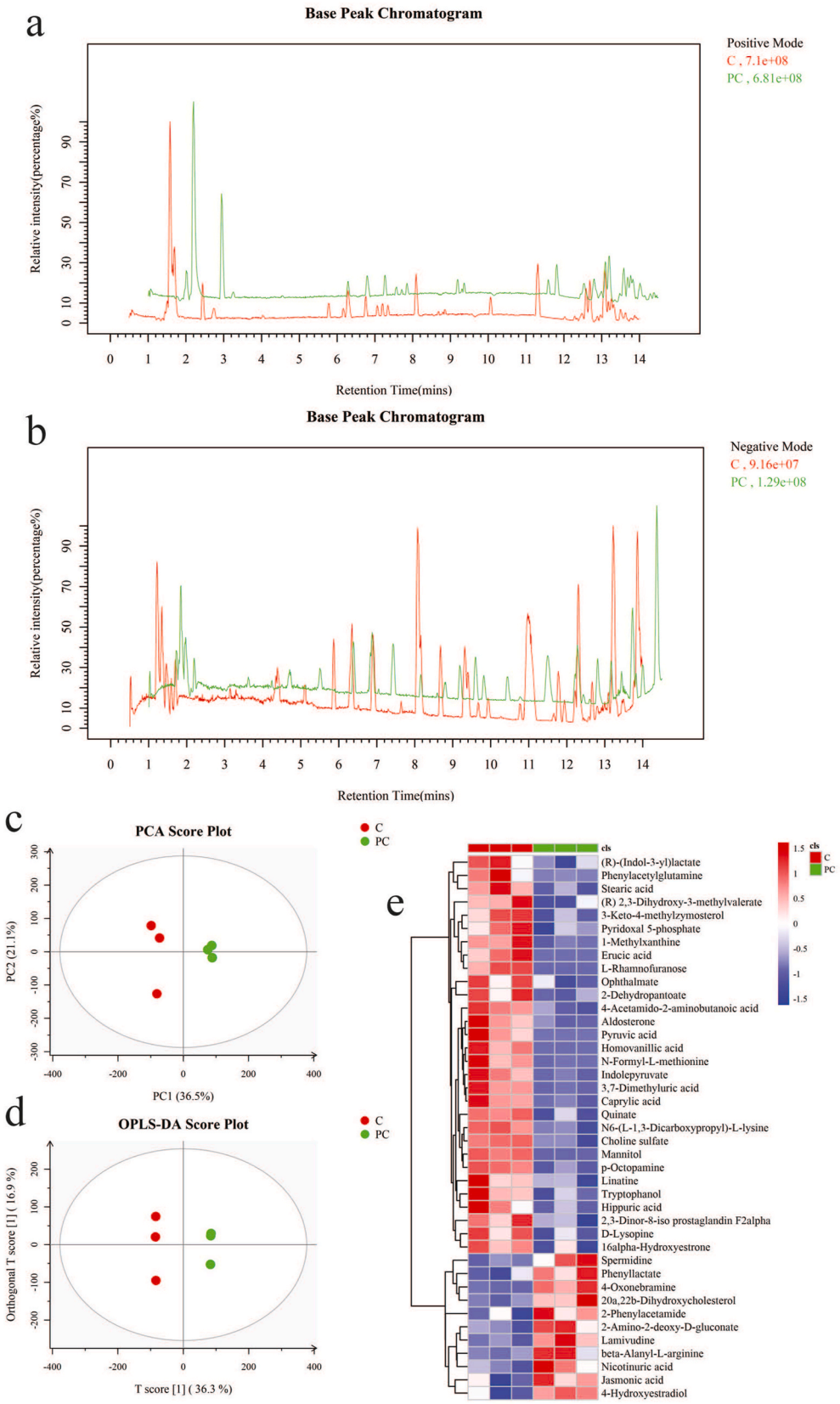

Fig. 4. Effect of the *L. plantarum*-pMG36e-GLP-1 on fecal metabolism of T2DM monkeys before (C) and after (PC) treatment. The ion chromatograms of the faeces sample in (a) positive and (b) negative modes; The dots in (c) PCA and (d) OPLS-DA indicating clear separation between the two periods; (e) Hierarchical clustering of heat map displaying the 41 significantly changed metabolites between two periods. The rows represent different specific metabolite, and the columns represent the individuals. The abundance level of metabolite changed with different colors. Red and blue mean increased and decreased levels of metabolites, respectively.

Table 2
Potential biomarkers identified in T2DM monkeys before (C) and after (PC) treated with *L. plantarum*-pMG36e-GLP-1.

No.	Metabolites	mean concentration		fold change (C/PC)
		C	PC	
1	Mannitol	69270951.81	1474006.72	46.995
2	*p*-Octopamine	20967456.61	12258399.75	1.711
3	Choline sulfate	40581979.17	21735102.29	1.867
4	L-Rhamnofuranose	683330704.10	215.68	3168300.000
5	N6-(L-1,3-Dicarboxypropyl)-L-lysine	33367994.29	11530183.68	2.894
6	4-Oxonebramine	1647792.00	12978509.50	0.127
7	4-Acetamido-2-aminobutanoic acid	25749261.03	11226789.93	2.294
8	Homovanillic acid	814701.09	215.68	3777.300
9	1-Methylxanthine	36901995.27	15474001.39	2.385
10	Erucic acid	162804312.30	215.68	754840.000
11	Lamivudine	124782739.60	310071688.50	0.402
12	Aldosterone	17835955.47	10861243.26	1.642
13	3-Keto-4-methylzymosterol	12723211.13	7084711.77	1.796
14	2-Dehydropantoate	26013384.61	15092305.99	1.724
15	2-Amino-2-deoxy-D-gluconate	22578141.77	62572654.29	0.361
16	2,3-Dinor-8-iso prostaglandin F2alpha	22348799.70	16759248.07	1.334
17	4-Hydroxyestradiol	391695969.30	531552176.60	0.737
18	20a,22b-Dihydroxycholesterol	23303358.55	37972436.51	0.614
19	Spermidine	158860735.60	803577367.40	0.198
20	Tryptophanol	7627140.94	4042589.22	1.887
21	D-Lysopine	28022369.93	10623311.99	2.638
22	(R) 2,3-Dihydroxy-3-methylvalerate	6677087.51	1179554.42	5.661
23	Pyridoxal 5-phosphate	21066618.49	14293125.63	1.474
24	Ophthalmate	34347091.10	18920789.71	1.815
25	Phenylacetylglutamine	127051559.60	5760702.20	22.055
26	Linatine	80983385.33	50006706.22	1.620
27	beta-Alanyl-L-arginine	2274890.07	7766763.78	0.293
28	Jasmonic acid	143468622.40	180381301.00	0.795
30	2-Phenylacetamide	38946014.84	70865550.57	0.550
31	3,7-Dimethyluric acid	8766071.51	2503871.42	3.501
32	Caprylic acid	5301240.10	998576.48	5.309
33	Indolepyruvate	31540363.61	21812926.82	1.446
34	N-Formyl-L-methionine	58695260.42	11627812.34	5.048
35	Quinate	9074725.22	3805647.54	2.385
36	Stearic acid	33217030.13	19338794.81	1.718
37	Pyruvic acid	21298227.64	250.41	85055.000
38	Phenyllactate	1137816.18	2036349.68	0.559
39	Nicotinuric acid	6369628.51	12842966.66	0.496
40	Hippuric acid	19912048.41	4286656.62	4.645
41	(R)-(Indol-3-yl) lactate	13261244.83	6026911.48	2.200

metabolite composition of faecal samples of monkeys was assessed using LC-MS-based metabolomic approaches. Overall, the results suggest that *L. plantarum*-pMG36e-GLP-1 exhibited a beneficial effects in T2DM monkeys.

Enteroendocrine L-cells liberate GLP-1, which increases the secretion of insulin and thus lowers the blood sugar level in a glucose-dependent manner [31]. GLP-1 analogues have been approved to treat T2DM. The glucose-lowering mechanisms of GLP-1 mainly include promoting the synthesis and secretion of insulin and also includes many others, like reducing the production of glucagon, affecting gastric emptying function, and suppressing appetite [32]. However, the short biological half-life of GLP-1 is identified as a key determinants delaying its application. Our recently published study showed an engineered strain MG1363-pMG36e-GLP-1 could decrease the FPG level and improve glucose intolerance in high fat diet-induced obese mice [24]. In this study, the treatment effect of a similar engineered strain *L. plantarum*-pMG36e-GLP-1 on spontaneous T2DM monkeys was evaluated. Compared to other animal models, rhesus monkey models are considered an excellent non-human model for basic and applied biomedical research because of the similarity in genetics and physiology with humans [33]. At the different stages of diabetes, the clinical characteristics and risk factors of rhesus monkey are like those of humans [29]. The normal glucose level should below 5.6 mmol/L, defined by the ADA, while the FPG level defined by the WHO is a little higher at 6.1 mmol/L [34,35]. Therefore, to investigate the hypoglycaemic effect of *L. plantarum*-pMG36e-GLP-1, three spontaneous T2DM rhesus monkeys with an FPG level higher than 6.1 mmol/L were enrolled in this study. Because of the differences in the characteristics and state of the animals and since GLP-1 may suppress appetite, some studies have shown that GLP-1 analogues decreased the FPG level while some increased the plasma insulin level [36,37]. The current study results clearly demonstrate that *L. plantarum*-pMG36e-GLP-1 led to a significant improvement in the FPG level of spontaneous T2DM monkeys after 2 weeks of administration with no effects on appetite.

The gut microbiota maintains a state of low-grade inflammation and is associated with T2DM as it plays a role in the progression of T2DM and metabolic disorders. In our previous study, the engineered strain MG1363-pMG36e-GLP-1 markedly increased the intestinal microbial diversity of obese mice [24]. In the present study, the intestinal flora composition was found to be different in monkeys after administration with a similar engineered bacterium, *L. plantarum*-pMG36e-GLP-1. The main changes in the gut microbiota linked to T2DM included a significant reduction in *Firmicutes* and an increase in *Bacteroidetes* [38]. Thus, the increased *Firmicutes* microbes that were also seen in the obese mice in our previous study [24] and the reduced *Bacteroidetes* microbes we observed in T2DM monkeys after *L. plantarum*-pMG36e-GLP-1 treatment indicate the benefits of these engineered strains on T2DM. Another study has shown that a reduction in butyrate-producing bacteria was the main change in the microbiota in T2DM patients [39], since butyrate stimulates the secretion of GLP-1, which improves insulin sensitivity and

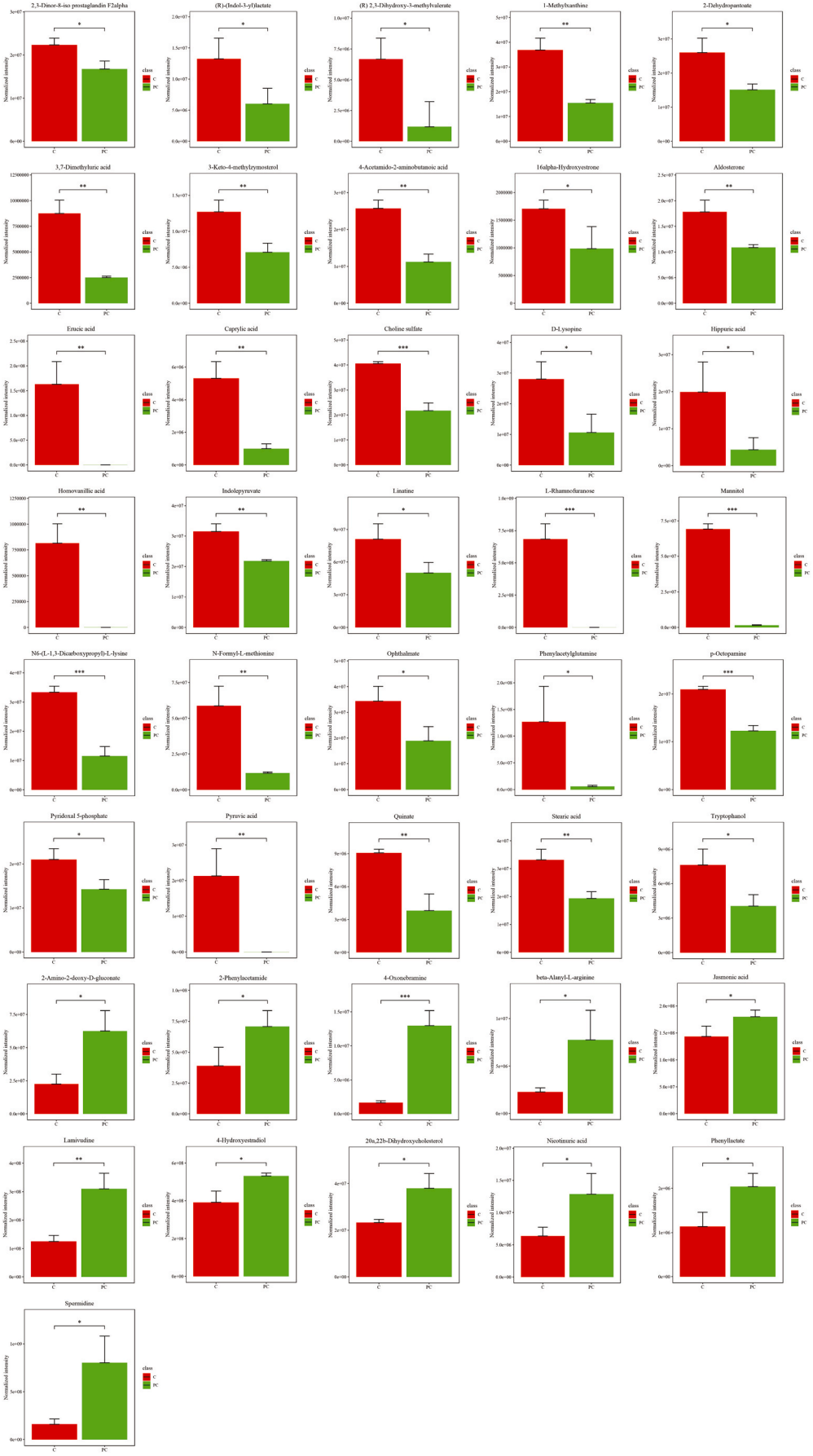

Fig. 5. The level of the 41 metabolites changed significantly in T2DM monkeys before (C) and after (PC) treatment with *L. plantarum*-pMG36e-GLP-1. Among these metabolites, the former 30 metabolites were significantly declined and the later 11 metabolites were obviously increased in monkeys after treatment. The ordinate represented the peak area, the red bar represents sample in the 0 week, and the green bar represents sample in the seventh week. * indicates a significant change between the samples before and after treatment: *p < 0.05, **p < 0.01.

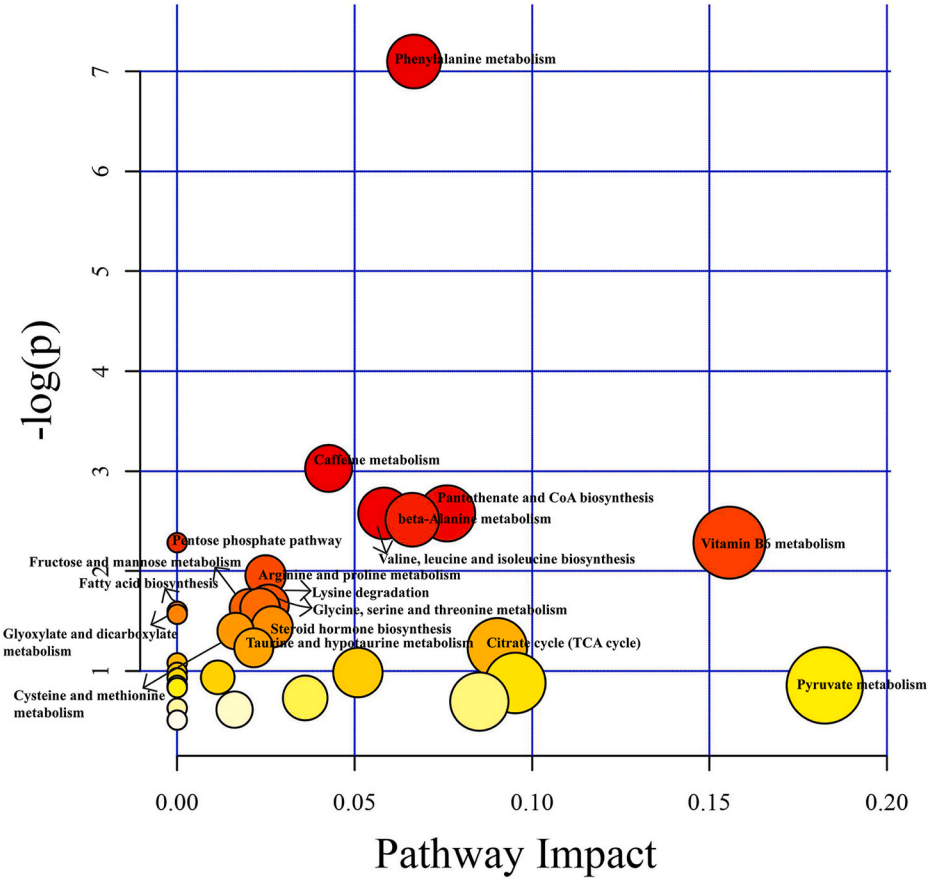

Fig. 6. The main metabolic pathways analysis of 41 metabolites changed according to the MetaboAnalyst. Node coloured based on p value and node radius determined based on pathway impact values. Some pathways that were affected, and the vitamin B6 metabolism (p = 0.005, impact 0.16) pathway was most heavily affected.

Table 3
Results (selected) from the pathway analysis.

Pathway	*p*-Value	Impact	
Vitamin B6 metabolism	0.005	0.156	Metabolism of cofactors
Pantothenate and CoA biosynthesis	0.003	0.076	and vitamins
Phenylalanine metabolism	7.97×10^{-8}	0.067	Amino acid metabolism
Valine, leucine, and isoleucine biosynthesis	0.003	0.058	
Arginine and proline metabolism	0.011	0.025	
Lysine degradation	0.022	0.026	
Glycine, serine, and threonine metabolism	0.024	0.023	
Cysteine and methionine metabolism	0.040	0.016	
Pentose phosphate pathway	0.005	0	Carbohydrate metabolism
Fructose and mannose metabolism	0.024	0.020	
Glyoxylate and dicarboxylate metabolism	0.027	0	
Fatty acid biosynthesis	0.025	0	Lipid metabolism
Steroid hormone biosynthesis	0.036	0.027	
Caffeine metabolism	9.40×10^{-4}	0.043	others
beta-Alanine metabolism	0.003	0.066	

secretion [40]. Our results show a significant enrichment of *Alistipes* in T2DM monkeys after the administration of *L. plantarum*-pMG36e-GLP-1 (Fig. 2h). *Alistipes* can produce short chain fatty acids (SCFA) like acetoacetate and butyrate and reverse the adverse impact of a high-fat diet [41]. It is quite possible that the enrichment of *Alistipes* is the immediate cause of decreased blood sugar in T2DM monkeys after taking the engineered bacteria. *Prevotella* is the main species linked to biosynthesis of branched-chain amino acids (BCAAs) with insulin resistance and has been found to induce insulin resistance [42]. The significantly lower level of *Prevotella* in T2DM monkeys after the administration of *L. plantarum*-pMG36e-GLP-1 compared to pre-treated monkeys in this study has been reported in relation to type 2 diabetes in mouse models [43] and to obesity in human adults [44,45].

Metagenomic sequencing has been used to comprehensively analyse the connection between microbial function and host physiology and explore the function of changed microbiota [46]. In the current study, analysis based on the KEGG database indicated that the changed intestinal bacteria in T2DM monkeys were closely related to metabolic disorders involving cofactors and vitamins, fructose and mannose, terpenoids and polyketides, as well as porphyrin and chlorophyll.

Faecal metabolome characterisation was used to further understand the microbial reactions to intestinal microbiota regulations. In this study, metabolite profiling in faeces was significantly different in T2DM monkeys before and after the administration of *L. plantarum*-pMG36e-GLP-1. The VIP [47] value was used as an important index reflecting the variable importance and to find potential biomarkers. A total of 41 potential metabolic biomarkers were identified, of which 30 metabolites were significantly reduced and 11 metabolites were markedly increased in monkeys after treatment (Fig. 5). The results of the metabolic pathway analysis indicated that 21 pathways with impact values > 0.01 were perturbed in monkeys (Fig. 6). The vitamin B6 metabolic pathway may be perturbed in monkeys in this study, although there were only two metabolites matched, i.e. pyridoxal 5-phosphate and pyruvic acid were

both reduced. Pyridoxal 5-phosphate is the biologically active form of vitamin B6; it is implicated in homocysteine metabolism and is an independent risk factor for cardiovascular disease [48]. In the US and China, the incidence of diabetes increased after vitamin fortification [49], while countries without fortification like Norway have a low prevalence of T2DM [50]. This increases the likelihood that obesity and T2DM may be related to excessive intake of B vitamins. It has been reported that excess vitamins B6 metabolism significantly increases plasma H_2O_2 levels [51]. The level of pyruvic acid is also higher in T2DM patients [52]. Pyruvate is an initial substrate for hepatic glucose production and plays a vital role in the pathogenesis of T2DM. Furthermore, in the current study, it was found that similar metabolites involved in lipid metabolism, like aldosterone [53] and caprylic acid [54] were higher in diabetes. Aldosterone may directly induce inflammation at the pancreatic β-cell level, compromising insulin secretion [55].

5. Conclusions

To sum up, the current study offers a new conception of the features of the engineered strain *L. plantarum*-pMG36e-GLP-1 in a spontaneous T2DM monkey model and discusses the impact on intestinal microbes and faecal metabolism. Therefore, *L. plantarum*-pMG36e-GLP-1 could become a new T2DM treatment.

Data availability

The datasets analysed in this study are available from the corresponding author on reasonable request. Raw sequences have been deposited in the GenBank under accession number PRJNA643924.

Ethics statement

Animal experiment was conducted in Nanchang University, and all procedures were established by the Animal Ethics Association of Nanchang University and were under the terms of the Regulations on the Administration of Laboratory Animals (China).

CRediT authorship contribution statement

Jie Luo: Project administration, lead, Writing – original draft, lead, Formal analysis, equal, Writing – review & editing, equal. **Hongfei Zhang:** Investigation, lead, Writing – review & editing, equal. **Jiachen Lu:** Investigation, supporting, Writing – review & editing, equal. **ChaoLin Ma:** Conceptualization, supporting, Writing – original draft, supporting, Writing – review & editing, equal, Funding acquisition, equal. **Tingtao Chen:** Conceptualization, lead, Writing – original draft, supporting, Formal analysis, equal, Writing – review & editing, equal, Funding acquisition, equal.

Declaration of competing interest

None declared.

Acknowledgements

We thank all the subjects and our colleagues who contributed to the study. This study was supported by the National Natural Science Foundation of China (grant no. 31760276, 31960171, 82060638), the Jiangxi Natural Science Foundation (grant no. 20171BAB204019, 20192ACB20022), and the “double 10-thousand plan” of Jiangxi Province (innovation and technology professionals as the high-end talent).

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