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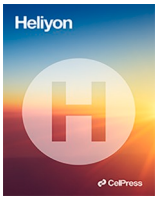

Research article

Comprehensive insights into berberine's hypoglycemic mechanisms: A focus on ileocecal microbiome in db/db mice

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ABSTRACT

The efficacy of berberine in managing diabetes through modulation of gut microbiome has been established through fecal sample analyses. However, relying solely on fecal materials constrains our comprehension of berberine's effects on diverse gastrointestinal locations. This study specifically explores the ileocecal region, a segment characterized by higher microbial diversity than fecal samples. Berberine exhibits a robust hypoglycemic impact by significantly reducing glucose levels in blood and urine. Beyond glycemic control, berberine ameliorates various diabetes-related symptoms in serum, including increased insulin and leptin, but decreased NEFA and MDA. Notably, berberine demonstrates liver-protective functions by alleviating oxidative stress and enhancing hepatic glycogen abundance. These outcomes prompted a high-throughput sequencing analysis of the ileocecal microbiome, revealing an augmentation of beneficial bacterial genera (four genera in the Lachnospiraceae family, *Erysipelatoclostridium*, and *Escherichia-Shigella*), along with a reduction in harmful bacterial genera (*Romboutsia*). Additionally, we predicted the impact of the ileocecal microbiome on clinically relevant factors associated with diabetes. These findings elucidate the multi-pathway mechanisms of berberine in treating T2D, underscoring its potential as a natural anti-diabetic agent or functional food, particularly through the modulation of the gut microbiota.

1. Introduction

Diabetes, a leading cause of global mortality, affects over 400 million people, with approximately 1.5 million annual deaths attributed to this disease [1,2]. The most prevalent form, type 2 diabetes (T2D), makes up majority of all instances [3].

Coptidis Rhizoma (Huanglian), a traditional Chinese herb, is renowned for its diverse pharmacological properties, including the management of diabetes, as recorded in the China Pharmacopoeia 2020. Berberine, the main active ingredients, is widely distributed in various plants and is notably effective in diabetes treatment [4]. A big clinical data analysis has shown a significant hypoglycemic effect of berberine [5]. Moreover, its efficacy has been demonstrated in both diabetic mouse [6,7] and rat models [8], with substantial research attention focused on its pharmacokinetics and pharmacological activities [9].

The low bioavailability of berberine in animals underscores the need for further exploration into its various significant biological

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functions. Emerging evidence suggests that berberine primarily targets gut microbiota, commonly called "hidden organ", which might play a pivotal role in its pharmacological effects. Berberine has been shown to manipulate gut microbiota in various pathological conditions, including obesity, hyperlipidemia, diabetes, cancer, and inflammatory [10,11].

Current reports highlighted a close relationship between T2D and intestinal flora [12]. The microbiota inhabits various host sites, including the gastroenteric and respiratory systems [13], and exhibit specific biogeographical distributions within different digestive tract locations [14]. Berberine's ability to modulate the gastrointestinal microbiome has been documented [15], particularly in diabetic db/db mice [16–18]. However, prior research primarily utilized fecal samples, limiting our understanding of berberine's impact on other gastrointestinal areas. Notably, the ileocecal region, situated in midst of gastrointestinal tract, boasts a microbiome of higher variety compared to that of fecal samples [14,19]. Here, in db/db mice, we explored berberine's effect on ileocecal microbiome.

2. Methods

2.1. Mice experiments

The db/db mice (male, 2 months old, among 40.00 g) and m/m mice (among 20.00 g) were bought from Changzhou Cavens Experimental Animal Co., Ltd. All animals raised under SPF conditions. After one week of acclimatization, they were assigned to model (DM), berberine (n = 7), and normal control (NC, n = 10) groups. The dose of berberine (Yuanye Bio-Technology Co., Ltd) administered was 0.2 g/kg·BW. The model and control groups given equal volume clean water. Treatments continued for 6 weeks.

2.2. Body mass and liver detection

Body mass was detected once a week. Upon completion of the experiment, after sedated with pentobarbital sodium (concentration 10 %, 50 mg/kg), livers were extracted. Liver index = liver (g): body (100 g) weight.

2.3. Blood index detection

The glucose level was monitored once a week from tail vein blood using a kit (ACON Biotech Co. Ltd). Oral glucose tolerance was conducted during the sixth week. Urine glucose levels were assessed with a reagent (Rongsheng).

Femoral artery blood was gathered and refrigerated at 4 °C, and serum was isolated through centrifuged 10 min at 3, 000 rpm. GSP levels were quantified by a dedicated reagent (Nanjing Jiancheng). Briefly, 10 μL of serum was added to a 220 μL system, and followed by colorimetric analysis using microplate reader at 530 nm. The serum insulin levels were assessed using an ELISA-based assay kit (CUSABIO). In short 100 μL of serum diluted 200 times was added to the reaction system, followed by microplate reader colorimetry at 530 nm.

Leptin levels were detected via an ELISA-based kit (CUSABIO). Briefly, 100 μL of serum diluted 5 times was added to the reaction system, followed by microplate reader colorimetry at 530 nm. The NEFA levels were detected via a kit fbought from Nanjing Jiancheng. In short, 4 μL serum was added to a 254 μL system, and colorimetry analysis was conducted using a microplate reader at 546 nm.

2.4. Histopathological and liver function analysis

Following treatment, the liver underwent histological analysis with HE and PAS staining. Liver samples (100 mg) were mixed with 1 ml of normal saline, and the homogenate was obtained by grinding with an electric homogenizer. Glycogen content, malondialdehyde (MDA), glutathione peroxidase (GSH-PX) superoxide dismutase (SOD), and catalase (CAT), were measured accordingly (Nanjing Jiancheng). For glycogen, colorimetric method was used, and 100 μL of liver homogenate diluted 5 times was added to the system, with absorbance read at 620 nm; for MDA, a TBA method was used, and 50 μL of liver homogenate was added to a 1000 μL system, 95 °C water bath for 40 min, with absorbance read at 532 nm; for CAT, a visible light method was used, and 10 μL of liver homogenate diluted 5 times was added to a 230 μL system, with absorbance read at 405 nm; for SOD, a hydroxylamine method was used, and 20 μL of liver homogenate diluted 50 times was added to a 240 μL system, with absorbance read at 450 nm; for GSH-PX, a colorimetric method was used, and 10 μL of liver homogenate diluted 40 times was added to the system, with absorbance read at 412 nm. All absorbance reading were performed using a microplate reader.

2.5. Gut microflora analysis

Total DNA of Bacterial from the ileocecal was isolated, V3∼V4 segments from 16s rRNA gene were examined on Illumina MiSeq using primers 338F and 806R.

2.6. Statistical analysis

Data was showed as mean ± standard deviation (SD). The Statistical examination was preformed employing independent sample t-tests in SPSS Statistics 21.0.

3. Results and discussion

3.1. Impact of berberine on body mass and hepatic index

Throughout experiment, every group of mice exhibited an increase in body mass. Neither starting nor terminal body mass showed difference in berberine group in contrast with DM groups (Table 1). However, the liver index was significantly higher in DM group in contrast with NC group. Hepatic index in berberine group did not differ from that in DM group, suggesting that berberine treatment have no notable impact on body mass or liver index.

3.2. Impact of berberine on diabetes indicators in blood and urine

Impact of berberine on improving diabetes were systematically evaluated. Weekly monitoring of blood glucose levels from tail vein samples revealed decreasing trend in berberine group from the second week onwards, reaching statistical significance reached by the sixth week (Fig. 1A). This trend was corroborated by measurements of serum glucose from the femoral artery, which were markedly reduced with the treatment of berberine ($p < 0.05$, Fig. 1B). Furthermore, urine glucose levels, an indicator of diabetes severity, were notably lower in berberine group (Fig. 1C).

Oral glucose tolerance testes reduced significantly in area under the blood glucose curve (AUC) in berberine group (Fig. 1D and E), providing additional evidence of berberine's effectiveness in bring down blood glucose levels. With the aim of assessing the prolonged-action on blood glucose stability, GSP levels were examined. The berberine group exhibited a significant reduction in GSP levels in contrast with DM group (Fig. 1F), affirming the sustained hypoglycemic impact of berberine.

Considering the aberrant insulin levels observed in T2D [20], it was noteworthy that insulin in berberine group exhibited a significant increase over those in DM group ($p < 0.05$, Fig. 1G), reaching levels similarly to the NC group. However, despite improvement in insulin levels, no differ in insulin resistance was observed between the berberine and DM groups.

This multifaceted analysis affirms the robust hypoglycemic impact of berberine in the context of db/db mice.

3.3. Impact of berberine on leptin level and NEFA level

The impact of berberine on leptin and NEFA was further investigated (Fig. 1H and I). Leptin, known for its role in inhibiting hyperglucagonemia in diabetes [21] and its intricate interplay with various factors affecting blood glucose levels [22–24], displayed a significant higher expression within DM group in contrast with NC group (Fig. 1H), in agreement with prior research [25]. Notably, the berberine group exhibited a significant elevation in leptin levels in contrast with the DM group (Fig. 1H), reinforcing positive interaction between leptin and berberine treatment. As illustrated in Fig. 1G, the similar modulation observed in the expression of leptin and insulin in response to berberine supports the notion of a positive interaction between these two factors [26]. While leptin is traditionally recognized as an adipokine that regulates food intake [27], our results suggest a novel role for leptin in db/db mice, which lack the leptin receptor. This lends credence to the hypothesis that berberine might reduce glucose via enhancing abundance as well as interplay of the two hormones.

Furthermore, NEFA ，a factor positively linked to the risk of diabetes [28,29], revealed a significant overexpression in DM group in contrast with NC group. Nevertheless, in berberine group, NEFA was markedly lower in contrast with DM group ($p < 0.05$, Fig. 1I). Down-regulation of NEFA by berberine potentially contributes to its glucose-lowering effect in db/db mice, providing additional insight into the multifaceted mechanisms through which berberine exerts its beneficial effects on glucose homeostasis.

3.4. Impact of berberine on liver indicators

The hepatic, a crucial controller of glucose balance, was examined to determine the impact of berberine on pathology, glycogen levels, and oxidative burden in the context of diabetes. Liver sections stained with HE and PAS revealed distinct patterns (Fig. 2A). The NC group exhibited regular hepatocyte morphology and well-organized arrangement, while the DM group displayed a notable proportion of liver cells with lipid accumulation and vacuoles. Remarkably, the berberine group showed less lipid accumulation and fewer vacuole-containing cells than DM group. These findings suggested that berberine mitigates hepatic damage associated with diabetes to a significant extent.

Elevated hepatic glycogen synthesis contributes to lowering blood glucose levels. PAS staining demonstrated increased glycogen content in DM group in contrast withNC group, consistent with previous reports [30,31]. Berberine further increased glycogen content (Fig. 2A). Quantitative analysis corroborated this observation, showing a significant elevation in glycogen levels in berberine group in

Table 1
Impact of berberine on body mass and hepatic index.

	Starting body weight (g)	Terminal body weight (g)	Hepatic index (g/100 g BW)
NC	20.80 ± 0.64[a]	26.20 ± 1.52[a]	3.97 ± 0.24[a]
DM	40.80 ± 4.64	48.40 ± 5.07	4.61 ± 0.64
Berberine	39.40 ± 1.92	47.70 ± 6.76	5.03 ± 0.23

[a] $p < 0.05$.

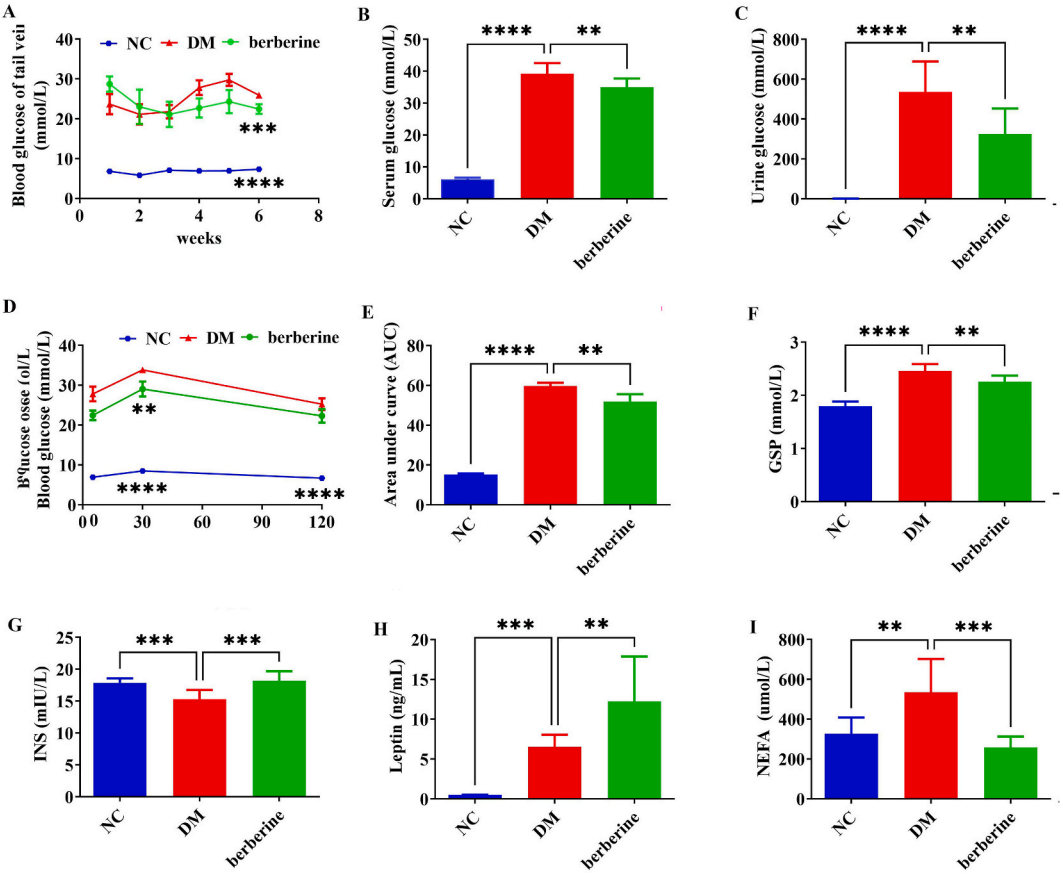

Fig. 1. Impact of berberine on diabetes. A, Glucose in blood; B, Glucose in serum; C, Glucose in urine; D, Oral glucose tolerance; E, Area under curve; F, glycated serum protein; G, insulin; H, Leptin; I, *nonesterified fatty acid*, *$p < 0.05$.

contrast with DM group (Fig. 2B). This aligns with previous reports indicating that improved diabetes in db/db mice is associated with increased glycogen levels [30].

Excessive superoxide anion free radicals generated during glucose oxidation under hyperglycemia contribute to heightened oxidative stress in diabetes. Previous studies have reported the antioxidant effect of demethylberberine [32]. To assess berberine's antioxidative capacity, we examined MDA, GSH-Px, CAT and SOD in liver. In DM group (Fig. 2C–F), CAT, GSH-Px, and SOD were markedly reduced, while MDA was elevated in contrast with NC group, implying elevated oxidative burden. Conversely, the berberine group exhibited a reduction of MDA and elevation of SOD as well as GSH-Px than DM group. Although not reaching statistical noteworthy, CAT also showed an upward trend. These results underscore the significant antioxidative stress effect of berberine.

3.5. *Impact of berberine on gut microbiota*

16s rRNA gene were detected in the ileocecal region. Alpha (Fig. 3A) and beta (Fig. 3B) diversity index were examined. Community coverage exceeded 99.86 % across all three groups, with no significant differences observed (Fig. 3A). Shannon index indicated a lower trend in community diversity ($p > 0.05$, Fig. 3A) in NC and berberine group than DM group. However, community richness, as represented by chao, ace, and sobs indexes, significantly reduced in NC and berberine group than DM group (Fig. 3A). These findings suggested that community diversity and richness were significantly elevated compared to the NC group, and berberine intervention effectively down-regulated these parameters.

The ileocecal bacterial composition in the berberine group exhibited clear separation from the DM group according to PCA analysis (Fig. 3B), indicating that berberine exposure induced alterations in bacterial composition in T2D mice.

To elucidate specific features of the microbiota among groups, LEfSe analysis was conducted (Fig. 3C). Notably abundant genera included *Romboutsia* in the DM group, *NK4A136* in the *Lachnospiraceae* genus, *Anaerostips*, an unclassified genus, and a norank genus from Lachnospiraceae family, *Erysipelatoclostridium*, and *Escherichia-Shigella* in the berberine group. *Romboutsia,* nearly absent in NC group, was raised in DM group but significantly reduced after berberine treatment. Previous studies also reported increased *Romboutsia* in T2D, decreasing after treatment [33,34]. No substantial distinction was detected in the *Lachnospiraceae* genus between NC and DM groups, and berberine effectively increased the richness of four genera from Lachnospiraceae family. Previous studies reported that the

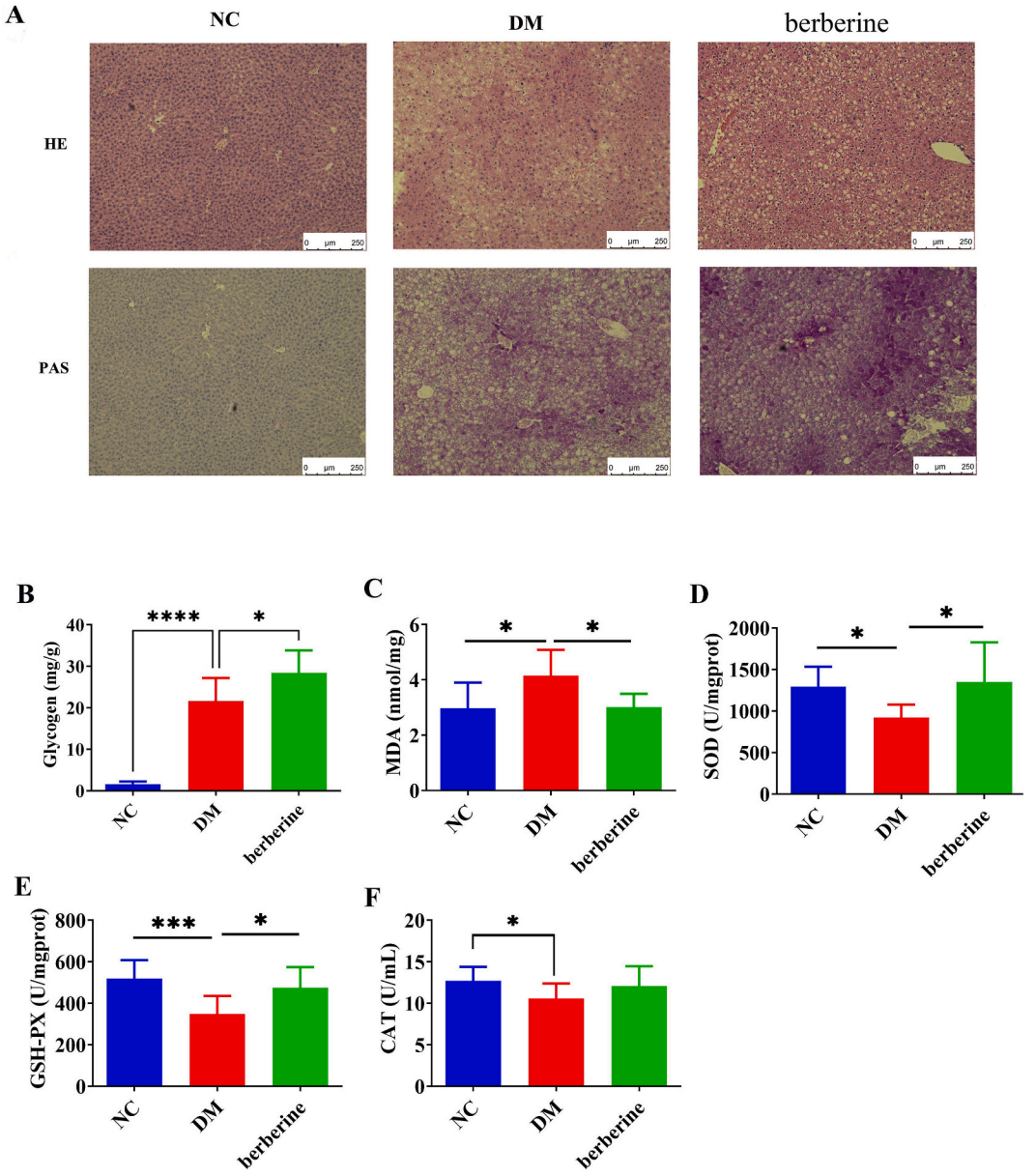

Fig. 2. Impact of berberine on liver. A, 10 × HE and PAS staining; B, Glycogen level; C, MDA; D, SOD; E, GSH-Px; F, CAT, $^{*}p < 0.05$.

Lachnospiraceae [35] and *Escherichia-Shigella* [36] genera exhibited a high proportion in diabetes.

OUT abundance was standardized using PICRUSt, and subsequent annotation with KEGG pathways was performed from level 1 to level 3.

Gut microbiome acts as a major contributor in metabolic system disorders of diabetes, as highlighted by Devaraj et al. in their study, emphasizing implication for obesity and diabetes [37]. Our data revealed that the overall metabolism status (level 1) was somewhat lower in DM group than NC group, while in berberine group, it higher significantly in comparison with DM group (Fig. 4). Specifically, at level 2, berberine significantly upregulated metabolic pathways mediated by gut flora (Fig. 4). Level 3 further elucidates more specific metabolic pathways, totaling 26 pathways (Fig. 4). Prior discovers have suggested that berberine holds promise to be a multi-target medicine for metabolic diseases [38]. Our results propose that the intestinal microbiota might constitute a crucial link in this context.

In addition to the metabolic system, organismal systems of diabetes also exhibit abnormalities. The main manifestation in db/db mice include the weakening of the immune system, endocrine system, and digestive system (Fig. 4). Berberine significantly up-regulate the immune and endocrine system but not the digestive system (Fig. 4). Specifically, berberine significantly up-regulated antigen processing and presentation, PPAR, NOD-like receptor, and insulin signaling pathways, while down-regulating the adipocytokine and

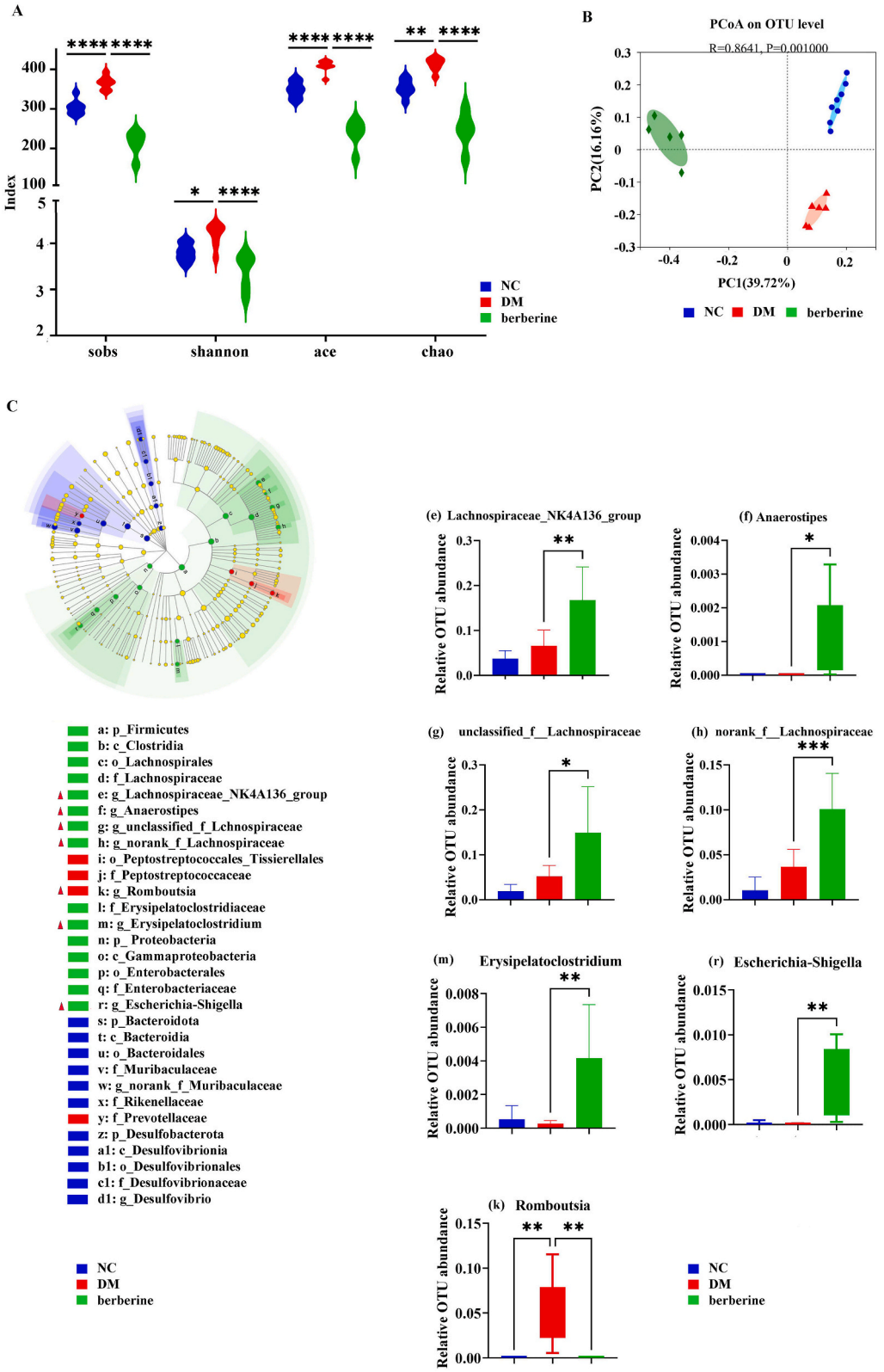

Fig. 3. Alpha diversity (A), PCA (B), and LEfSe among groups, *$p < 0.05$.

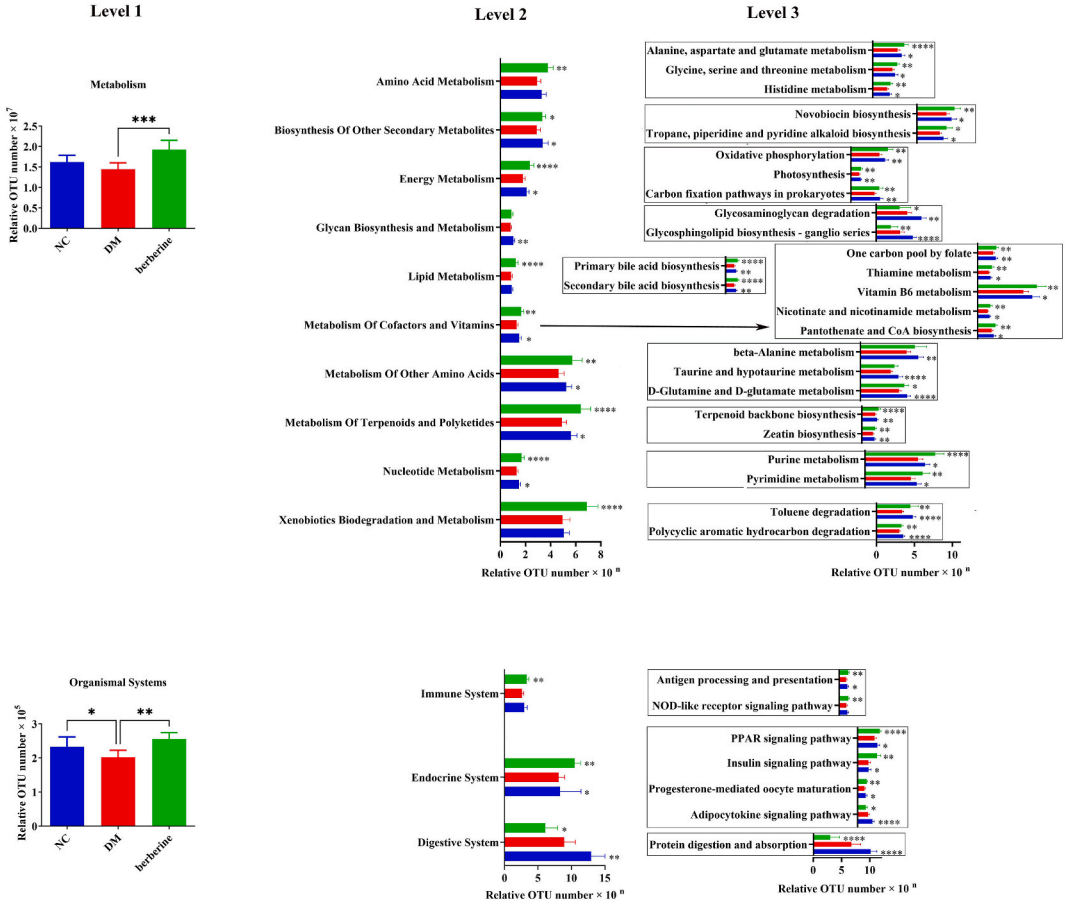

Fig. 4. Impact of berberine on KEGG pathway, $*p < 0.05$.

protein digestion and absorption pathway (Fig. 4). Different pan PPAR agonists can be employed in the treatment of T2D [39]. Berberine might activate the PPAR signaling pathway through intestinal flora to treat diabetes. Insulin, which plays a pivotal role in diabetes, is regulated by berberine, with insulin resistance being a significant factor to etiology and pathogenesis of T2D, [40]. Our results showed that berberine up-regulated insulin and the microbiota involved in insulin signaling pathways, although the impact of berberine on insulin resistance was not significant. The adipocytokine signaling pathway, which has an impact on the progression of T2D [41], might be a target of berberine in treating T2D.

To further comprehend how berberine affects diabetes through the ileocecal Microbiome, Redundancy Analysis (RDA) was carried out to investigate correlation of gut microbiota and clinical factors. The outcomes revealed significant correlations (Fig. 5A, Table 2) between clinical factors such as GSP, glucose, leptin, INS, NEFA in serum, and glycogen, MDA, SOD, CAT, GSH-Px abundance in the liver with ileocecal microbiota composition. A correlation heatmap was generated to study the link of the afore mentioned clinical factors and ileocecal microbiota (Fig. 5B). The results indicated that every clinical factor has a certain effect on at least one genus of flora. In the DM group, the *Romboutsia* genus exhibited a marked increase and was positively correlated with blood and urine glucose levels. Additionally, it exhibited positive relationship with GSP and NEFA in serum, while a negative relationship with INS in serum (Fig. 5A). *Romboutsia* was positively associated with MDA but inversely related toGSH-PX and SOD in liver (Fig. 5B). These findings suggested that *Romboutsia* genus is closely related to diabetes in db/db mice, and potential target of berberine's hypoglycemic effect. The *NK4A136* group in *Lachnospiraceae* genus, an unclassified genus, and a norank genus in Lachnospiraceae family were associated with glycogen in liver (Fig. 5B), indicating their potential roles in hepatic glycogen synthesis.

4. Conclusions

To summary, this study comprehensively explored the multi-pathway effects of berberine in improving T2D in db/db mice, with a particular focus on its regulatory mechanisms on the ileocecal microbiota. Berberine significantly reduced glucose in blood as well as urine, demonstrating a potent hypoglycemic effect. A comprehensive assessment of diabetes-related symptoms revealed that berberine not only increased insulin and leptin while reducing NEFA as well as MDA but also decreased urine glucose levels. Additionally, berberine exhibited a liver-protective function by alleviating oxidative stress in the liver and significantly increasing hepatic glycogen

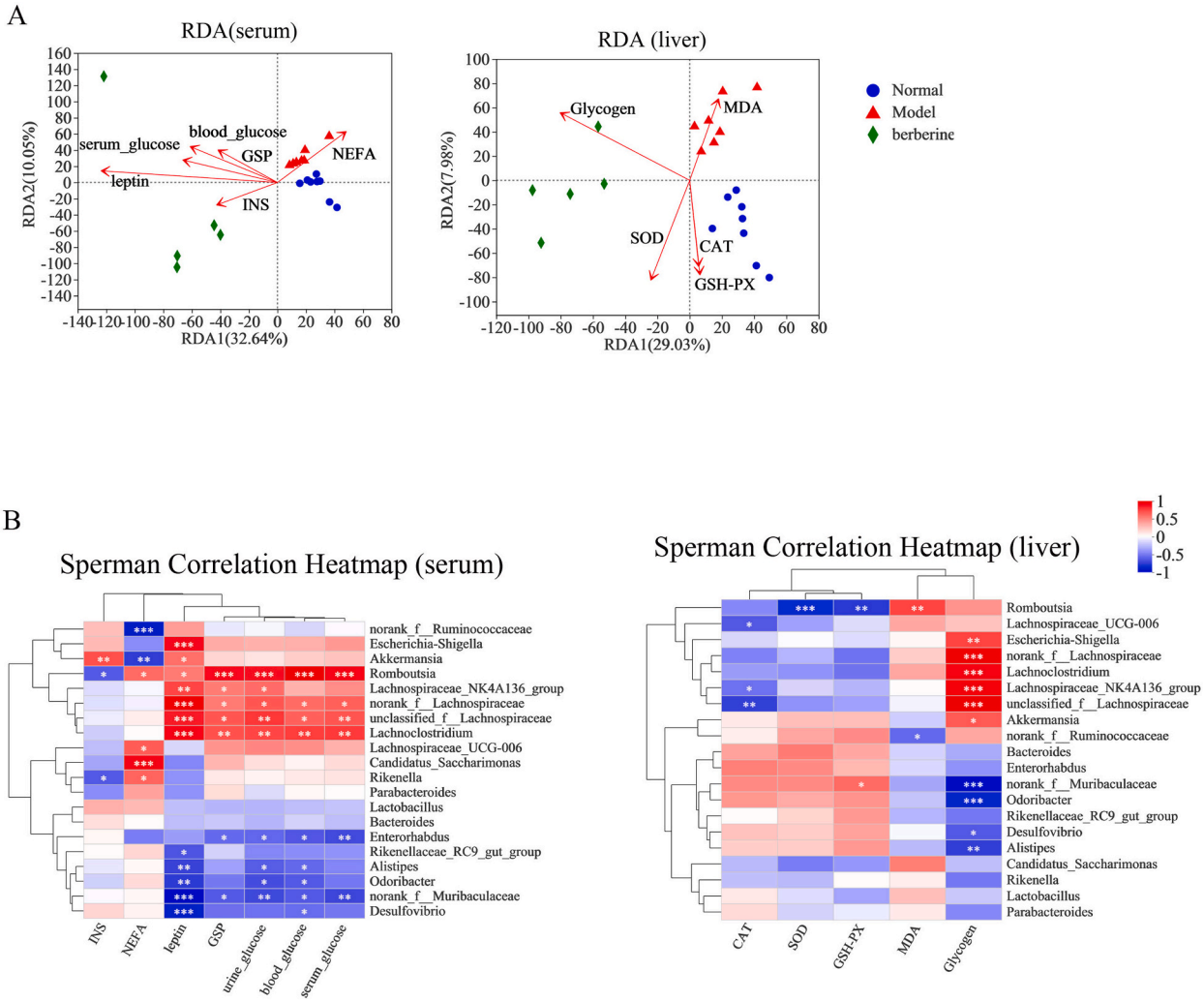

Fig. 5. The RDA and heatmap analysis of clinical factors and flora. (A) RDA analysis (B) Spearman correlation heatmap. *$p < 0.05$.

Table 2
RDA analysis.

Clinical factor	Sample	RDA1	RDA2	r2	P values
blood glucose	Tail veil blood	−0.8665	0.4992	0.2675	0.037
serum glucose	Serum	−0.9331	0.3596	0.2501	0.051
GSP		−0.813	0.5822	0.1476	0.234
INS		−0.933	−0.3599	0.0966	0.442
leptin		−0.9833	0.1821	0.7599	0.001
NEFA		0.7233	0.6906	0.2097	0.138
SOD	Liver	−0.28	−0.96	0.6081	0.002
GSH-PX		0.0698	−0.9976	0.5015	0.005
CAT		0.2571	0.9664	0.3972	0.014
MDA		0.0669	−0.9978	0.4144	0.009
Glycogen		−0.8145	0.5802	0.778	0.001

abundance. The 16S rRNA results exhibited that berberine treatment enhanced the abundance of beneficial bacterial genus (four genera from Lachnospiraceae family, *Erysipelatoclostridium* and *Escherichia-Shigella*) while reducing harmful bacterial genera (*Romboutsia*).

As far as we are aware, this study might represent the first exploration to delve into the hypoglycemic effects and mechanisms of berberine in ameliorating T2D via multiple pathways, such as the modulation of ileocecal microflora. These findings establish a scientific foundation for utilizing berberine as a natural anti-diabetic medicine or functional substance, highlighting its broad-

spectrum hypoglycemic effects, notably through modulation of the gut microbiota.

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Ethics statement

Animal experiments received approval from the Laboratory Animal Welfare Ethics Committee of Zhejiang Academy of Traditional Chinese Medicine (Approval No: 2021-035).

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Xuan Chen: Writing – original draft, Visualization, Validation, Software, Methodology, Data curation, Conceptualization. **Xi-yu Mei:** Validation, Methodology, Data curation. **Ze-ming Ren:** Validation, Methodology, Data curation. **Si-si Chen:** Validation, Methodology, Data curation. **Ye-ling Tong:** Validation, Methodology, Data curation. **Cui-ping Zhang:** Validation, Methodology, Data curation. **Jia Chen:** Validation, Methodology, Data curation. **Guan-hai Dai:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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