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Semaglutide alters gut microbiota and improves NAFLD in db/db mice

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease associated with type 2 diabetes mellitus (T2D). NAFLD can progress to nonalcoholic steatohepatitis (NASH), cirrhosis, and even cancer, all of which have a very poor prognosis. Semaglutide, a novel glucagon-like peptide-1 (GLP-1) receptor agonist, has been recognized as a specific drug for the treatment of diabetes. In this study, we used a gene mutation mouse model (db/db mice) to investigate the potential liver-improving effects of semaglutide. The results showed that semaglutide improved lipid levels and glucose metabolism in db/db mice. HE staining and oil red staining showed alleviation of liver damage and reduction of hepatic lipid deposition after injection of semaglutide. In addition, semaglutide also improved the integrity of gut barrier and altered gut microbiota, especially Alloprevotella, Alistpes, Ligilactobacillus and Lactobacillus. In summary, our findings validate that semaglutide induces modifications in the composition of the gut microbiota and ameliorates NAFLD, positioning it as a promising therapeutic candidate for addressing hepatic steatosis and associated inflammation.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) refers to a multi-etiological clinical syndrome with pathological fat deposition and degeneration, inflammation, and fibrosis. It can be divided into simple fatty liver disease and nonalcoholic steatohepatitis (NASH) according to its severity of the NAFLD [1]. Nowadays, most of the people's exercise is far from keeping up with the rapid improvement of living standards, coupled with irregular eating habits, so the incidence of NAFLD is increasing year by year. A recent study showed that The global prevalence of NAFLD is approximately 25%, and it is increasing rapidly [2,3]. In addition, people who were overweight or obese and who smoked had a higher incidence compared with those who were of normal weight and did not smoke. As the disease progresses, NAFLD can progress from simple steatosis to NASH, cirrhosis, and even cancer [4]. For people with simple fatty liver disease, advice on healthy diet and exercise is all that is needed. For obese NAFLD patients, the goal of lifestyle intervention is to achieve a weight loss of 7–10%, which can improve liver serological and histological markers. However, due to the complex heterogeneity of NAFLD, limitations of therapeutic targets, drug safety and other factors, these measures can only prevent or minimize the progression of NAFLD, but there is no way to treat an established NAFLD [5].

Semaglutide is a new GLP-1 receptor agonist. GLP-1 receptor agonist is recognized as a drug that benefits from type 2 diabetes and cardiovascular disease in current guidelines at home and abroad and has been proved to be effective for NAFLD patients with diabetes [6,7]. However, the specific mechanism by which GLP-1 agonists improve NAFLD remains unclear. Studies have shown that GLP-1 may play a role in the intestinal epithelium, and these roles are associated with changes in the gut microbiota [8,9]. Alterations in the gut microbiota may affect the morphology and function of the gastrointestinal tract and ultimately the degree of liver damage in patients with NAFLD [10,11]. The disorder of intestinal flora can promote the occurrence, development, and even deterioration and carcinogenesis of NAFLD. Therefore, how to use intestinal flora to change or block the progression of NAFLD, and try to find bacteria and bacterial products as potential targets, has become the key to the intervention and treatment of NAFLD. Whether some specific microbiota is associated with the occurrence and development of NAFLD, and whether it can be used as an effective treatment or target, is not yet clear in the mechanism. Gut microbiota dysfunction has been

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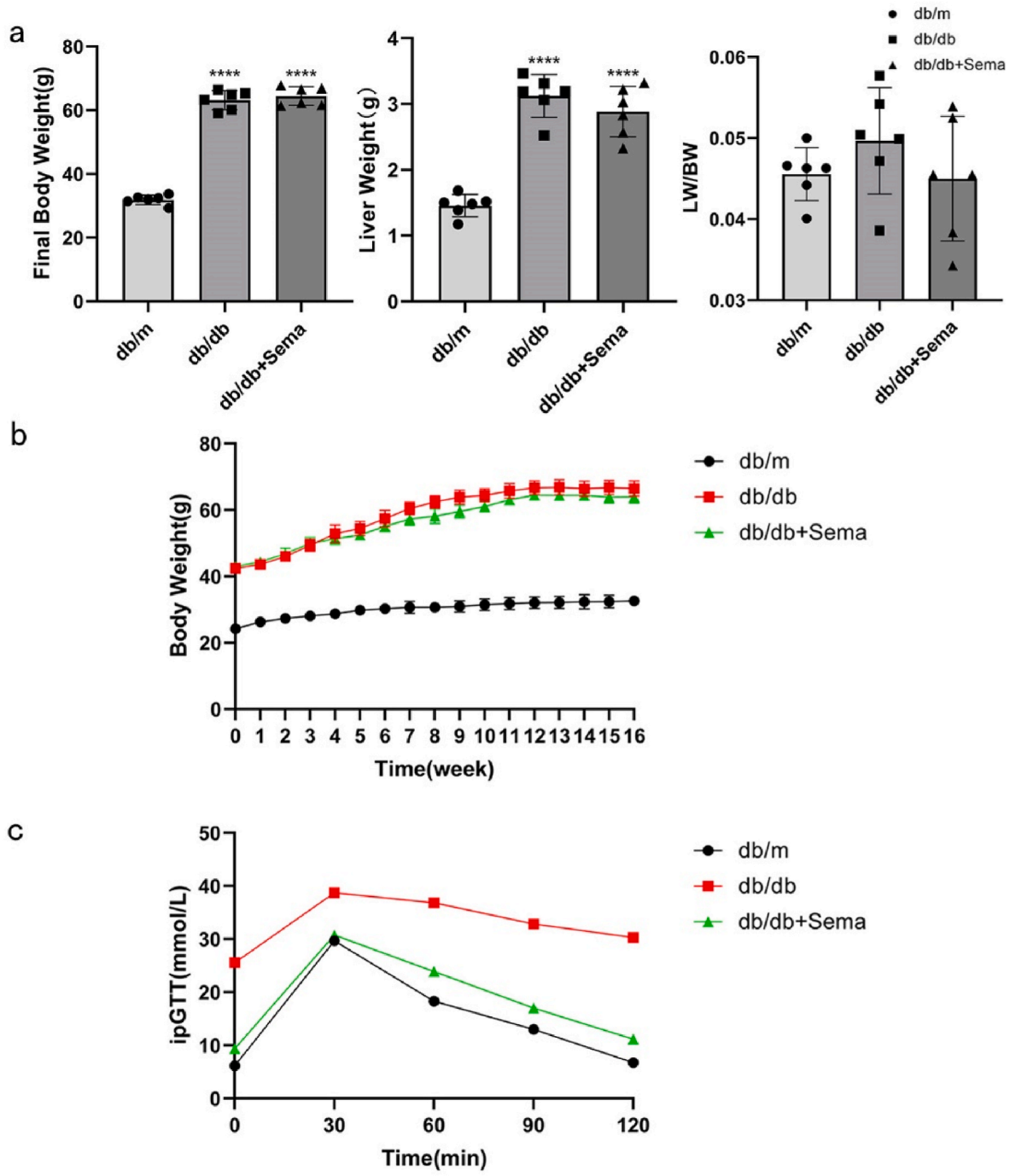

Fig. 1. Changes of body weight and liver weight in mice and ipgtt experiment. (a) Final body weight and liver weight of mice at 24 weeks ($p < 0.0001$). (b) Body weight status of mice per week. (c) Changes in blood glucose over time in mice after intraperitoneal injection of glucose.

recognized as one of the risk factors for NAFLD, so the main objective of this study was to investigate the effects of semaglutide treatment on the degree of liver damage and gut microbiota in a mouse model of inherited obesity.

2. Materials and methods

2.1. Mice

Male db/m mice and db/db mice (six weeks old) were purchased from Shulaibao Biotech Technology Co. Ltd (Wuhan, Hubei). All mice fed in specific-pathogen free (SPF)-room under the 12 h alternation cycle of day/night, temperature of (24 ± 2) °C, and relative humidity of (60 ± 10) %. After two weeks of acclimation, db/db mice were randomly divided into two groups. One group received once-weekly intraperitoneal injection of semaglutide (0.22 mg/kg once every 3 days) and the other received an equal volume of saline [12]. Their body weight and blood glucose were monitored weekly for 16 weeks. Mice were anesthetized by intraperitoneal injection of pentobarbital sodium (40 mg/kg) and sacrificed on the 24 weeks. Blood and liver samples were collected and stored immediately after execution for subsequent analysis. The animal study was reviewed and approved by The Animal Ethics Committee of Renmin Hospital of Wuhan University (The IACUC approval number: WDRM20211203).

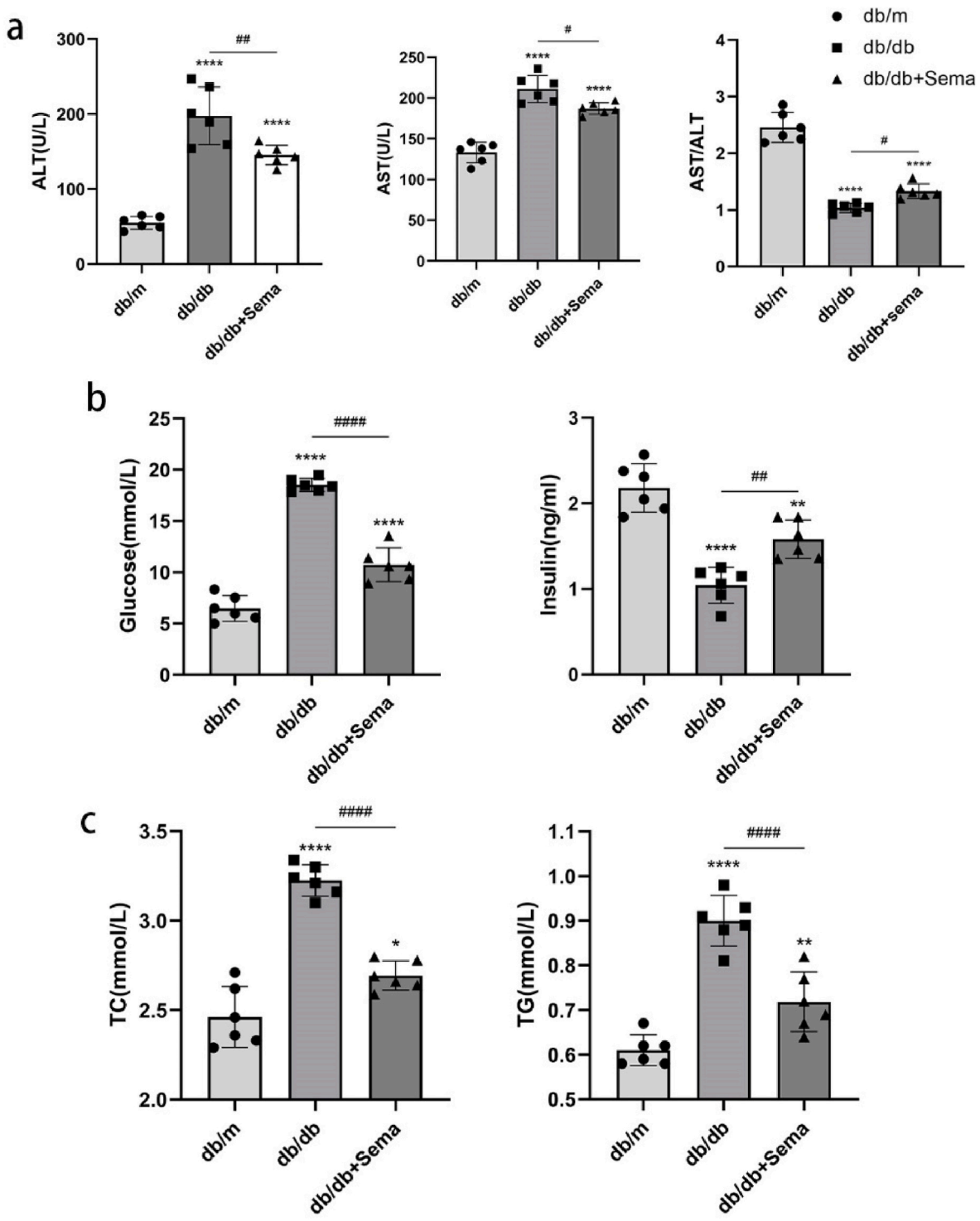

Fig. 2. Semaglutide alleviated the blood lipid profile and liver damage in db/db mice. (a) Semaglutide reduced liver damage in dbdb mice. (b) Semaglutide improved glucose metabolism in dbdb mice. (c) Semaglutide reduced the levels of TC and TG in dbdb mice (*$p < 0.05$,**$p < 0.01$,****$p < 0.0001$).

2.2. Intraperitoneal glucose tolerance test

All mice were fasted for 16 h and then given an intraperitoneal injection of glucose solution (2 g/kg). Blood from the tail vein was collected at 0, 30, 60, 90 and 120 min for glucose measurement.

2.3. Blood analyses

Blood samples were collected and sent to Renmin Hospital of Wuhan University (Hubei Wuhan, China) for measurement of glucose, ALT, AST, total cholesterol, and triglyceride levels.

2.4. HE staining and oil red O staining

Portions of the liver and cleaned small intestine were immediately placed in tissue fixative, dehydrated and embedded in paraffin. Hematoxylin-eosin (HE) and Oil red O staining were performed by Thermo Fisher Scientific Inc (Shanghai, China). The liver lipid

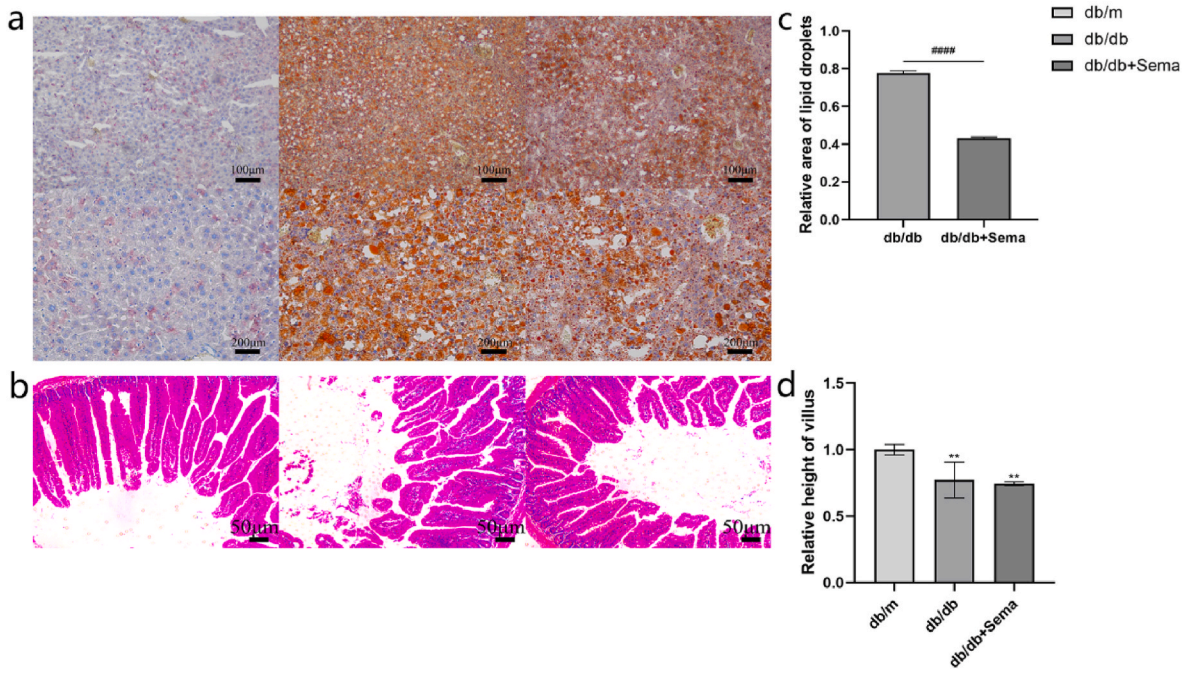

Fig. 3. Effects of semaglutide on liver histology and Intestinal villi changes in NAFLD model. (a) Representative images of lipid deposition stained with Oil Red O. (b) Representative images of Intestinal villi stained with HE staining. (c) Quantitative analysis of lipid deposition by Image J. (d) Quantitative analysis of Intestinal villi height by Image J (**p < 0.01,****p < 0.0001). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

deposition was quantified by Image-pro plus 6.0 software (Media Cybermetrics, Rockville, MD, USA).

2.5. Microbiota analysis

In order to ensure the accuracy and reliability of sequencing data from the source, we have rigorous and reliable sample detection and quality control processes from DNA extraction to sequencing. The quality of samples is strictly controlled in every link to ensure the authenticity and reliability of sequencing data. Sequencing of the 16S rRNA gene was performed on an Illumina MiSeq, and data obtained with Illumina MiSeq amplicon sequencing was analyzed with QIIME2 for the microbiota profiles. The sequencing analysis was performed by Biotree Biotechnology Co., Ltd (Shanghai, China).

2.6. Statistical analysis

The data results were analyzed using GraphPad Prism v8.02 software (GraphPad Software, La Jolla, CA, USA). Graphs represent mean + standard error of the mean (SEM). Statistically significance was accepted at $P < 0.05$ level (*), $P < 0.01$ level (**), $P < 0.001$ level (***), $P < 0.0001$ level (****) , $P > 0.05$ level (ns).

3. Results

3.1. Semaglutide demonstrated a notable enhancement in glucose metabolism, albeit with limited impact on ameliorating obesity in the db/db mice

To investigate the role of Semaglutide in the treatment of NAFLD, we chose db/db mice, a common genetic defect model of NAFLD. Our results showed that the body weight of db/db mice was significantly higher than that of db/m mice from the beginning to week 16 with or without semaglutide injection (Fig. 1b). Similarly, these two groups also had significantly heavier livers than the db/m group. However, semaglutide did not reduce body weight and liver weight in db/db mice (Fig. 1a). The results of IPTGG experiment showed that the glucose tolerance of db/db mice was obviously impaired, and Semaglutide could reduce the degree of impairment (Fig. 1c). In conclusion, semaglutide did not reduce body weight but improved blood glucose levels in db/db mice.

3.2. Semaglutide ameliorated liver injury and glucose metabolism and also reduced lipid levels in db/db mice

We found significant liver damage and dyslipidemia in db/db mice at week 24. The results showed that the levels of ALT, AST, ALT/AST, TC and TG were significantly increased in db/db mice. However, after injection of semaglutide, the levels of ALT, AST, AST/ALT, TC and TG showed a significant reduction (Fig. 2a and c). What's more, semaglutide also improved glucose metabolism in db/db mice. The blood glucose of db/db group was significantly higher than that of control group and the insulin concentration was significantly lower (Fig. 2b). Semaglutide can improve blood glucose metabolism level of db/db to some extent.

3.3. Semaglutide effectively reversed characteristics associated with NAFLD while also contributing to the preservation of intestinal barrier integrity

Oil red O staining of liver tissue showed that there was significant lipid deposition in the liver tissue of db/db mice, and semaglutide reduced the area of lipid deposition (Fig. 3a) , and the difference was statistically significant (Fig. 3c). HE staining of small intestine stated that the small intestinal villi were significantly shortened in db/db mice (Fig. 3d). Semaglutide did not reverse this trend, but it significantly improved the reality that the integrity of the small intestinal villi was impaired in db/db mice (Fig. 3b).

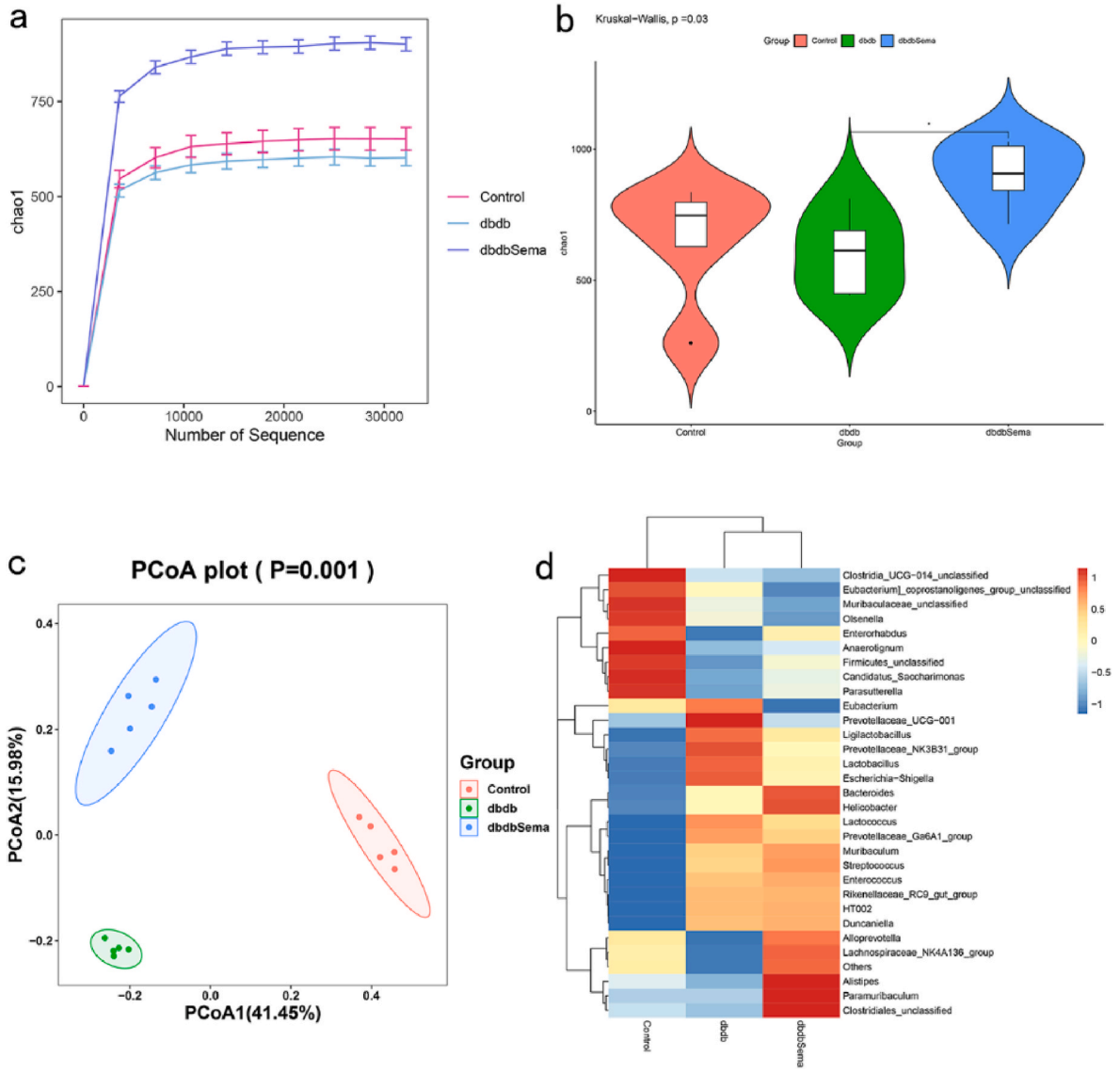

Fig. 4. 16s sequencing results showed that semaglutide altered the gut microbiota of db/db mice. (a) (b) (c) Semaglutide affected the gut microbiota richness, α-diversity and β-diversity of dbdb mice.(d) Heat map showed a significant change in the abundance of reads between db/db and semaglutide treated groups.

3.4. Semaglutide led to a notable enhancement in gut microbial diversity in db/db mice

To assess the effect of semaglutide treatment on the diversity of the gut microbiota, we performed 16S rDNA sequencing analysis of bacteria extracted from the feces of each group of mice.

The rarefaction curves estimate species richness in the environment by simulating the process of resampling and observing the trend of species change. The differences in species diversity between samples can be visualized by comparing the dilution curves of different samples. Our dilution curves tended to be flat, indicating that the amount of sequencing data was gradually reasonable (Fig. 4a). Sequencing results showed that the alpha diversity of gut microbiota was significantly reduced in db/db mice, while semaglutide treatment significantly increased the alpha diversity of gut microbiota (Fig. 4b). Similarly, PCoA results also revealed clear differences in the beta diversity of gut microbiota among the three groups (Fig. 4c).

To represent the most prominent genus level, we plotted the metagenomic profile as a heat map. The results showed a significant change in the abundance of reads between db/db and semaglutide treated groups. In order to judge the abundance of each different species in different groups, all species at the genus level were analyzed for differences separately, and the top 10 species with p value less than 0.05 were screened for bar graphs. LDA and LEfSe were used to find the species with significant differences in abundance between groups and rank them according to the effect size. The LEfSe analysis plot was more intuitive to show the species with significant differences at all levels between groups.

Heat map results showed that there were significant changes in *Alloprevotella* , *Alistpes* , *Ligilactobacillus* and *Lactobacillus* in db/db group and semaglutide treatment group (Fig. 4d). These were also confirmed by the results of the significant difference analysis (Fig. 5a). This result was also supported by the LDA score, *Alloprevotella* and *Alistpes* increased while *Ligilactobacillus* and *Lactobacillus* decreased after treatment with semaglutide injection (Fig. 5b and c).

4. Discussion

Semaglutide, as the most recent anti-diabetic drug, did not reduce body weight or liver weight in db/db mice as expected in our study. But the ability of semaglutide to regulate blood glucose and improve glucose metabolism is undeniable. After the injection of semaglutide, the db/db

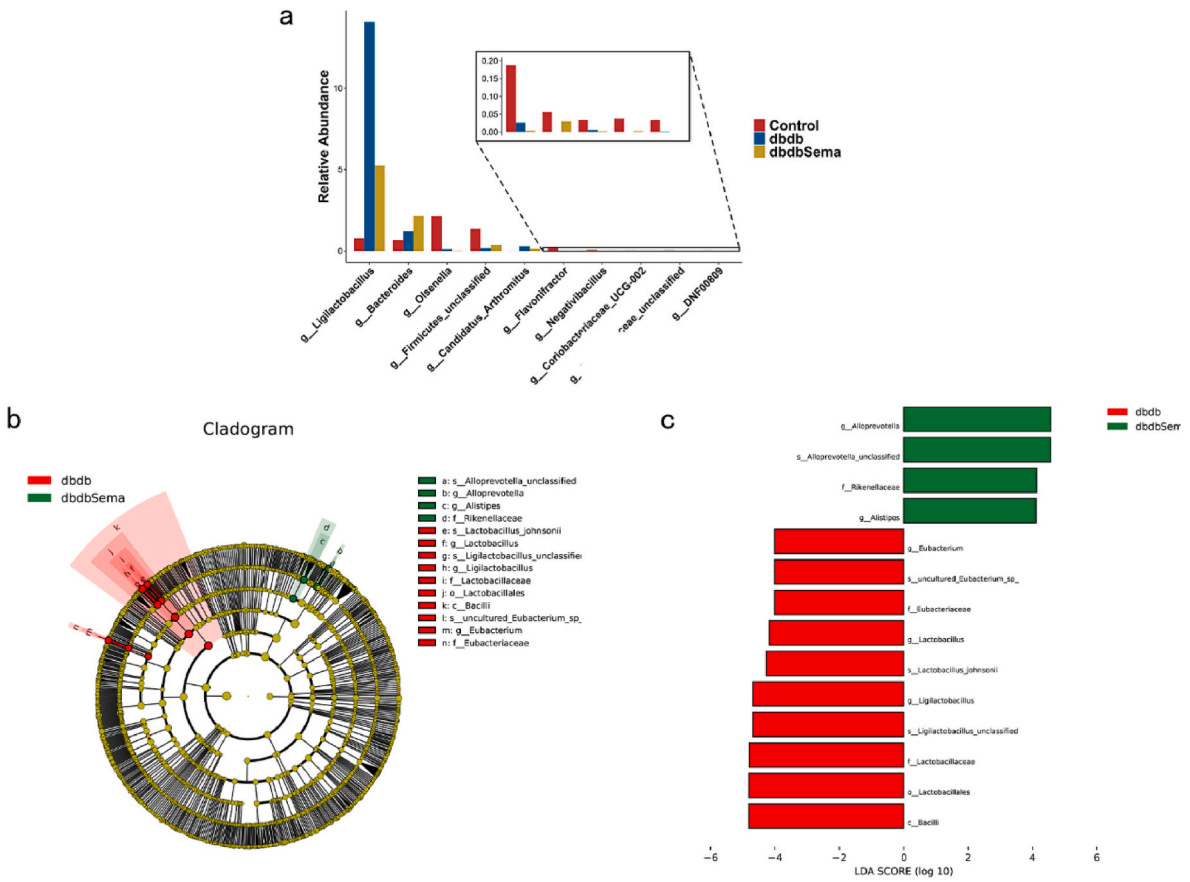

Fig. 5. Genera with significant differences after semaglutide treatment were analyzed. (a) Significant difference analysis identified the ten most divergent genera. (b) (c) LDA scores are displayed of the differentially abundant genera taxa with statistical and biological significance, ranked according to the effect size.

mice improved insulin secretion deficiency and reduced blood glucose significantly. What's more, the blood lipid content was also significantly reduced.

Semaglutide also significantly reduced lipid deposition in the liver and repaired liver damage. The ALT and AST levels were significantly reduced in db/db mice after semaglutide treatment. The HE and oil red staining of the liver also suggested that semaglutide could improve liver inflammation and lipid deposition.

Above all, although semaglutide did not reduce the body weight and liver weight of db/db mice, it did improve blood glucose metabolism and reduce blood lipid levels、liver damage and liver lipid deposition. In conclusion, semaglutide has a certain improvement on NAFLD.

These changes are well understood, since semaglutide has potent glucose-lowering and metabolism-improving effects. The reduction of blood glucose can be an important reason for the decrease of blood lipids, the improvement of liver lipid deposition and liver injury. We wondered if there were other causes of these alterations. The results of HE staining of the small intestine caught our attention. We found that the intestinal villi of db/db mice were significantly disorganized, and the integrity was damaged to varying degrees. However, after semaglutide treatment, the above conditions were significantly improved. Previous studies have shown that changes in gut microbes may be an important factor leading to gastrointestinal barrier disorders and intestinal inflammation [13,14]. Consequently, our objective was to explore the impact of semaglutide treatment on the gut microbiota composition in db/db mice. This led us to consider alternative explanations for the therapeutic effects of semaglutide. To achieve this, we collected fecal samples from the mice to conduct a comprehensive analysis of the gut microbiota.

The results of gut microbiota indeed confirmed our conjecture. Gut dysbiosis has been shown to be a contributing factor to the progression of obesity, insulin resistance, and fatty liver. The gut microbiota of db/db mice was significantly altered after injection with semaglutide. *Alloprevotella* and *Alistpes* increased while *Ligilactobacillus* and *Lactobacillus* decreased, and this change was significant and meaningful. A growing number of studies have confirmed the ability of certain intestinal microorganisms to improve liver fat accumulation and repair the intestinal barrier [15,16].

Alloprevotella and *Alistpes* are SCFAs-producing (short chain fatty acids) microbes, which might at least in part contribute to the anti-inflammatory and cholesterol-lowering. What's more, lots of studies have found that SCFAs-producing microbes can alleviate many intestinal inflammatory diseases and even inhibit the development of gastrointestinal tumors [17,18]. *Desulfovibrio vulgaris*, one of the SCFAs-producing microbes was reported to have the ability to attenuate hepatic steatosis and its related metabolic disorders [19]. SCFAs treatment significantly reduced serum ALT and AST levels, the number of lipid droplets, and triglyceride and cholesterol levels in the liver of mice. SCFAs also reduced MCD-induced hepatic aggregation of macrophages and proinflammatory responses. SCFAs can induce the expression of fatty acid oxidation related genes in liver and hepatocytes cultured in vitro [20]. As for *Ligilactobacillus* and *Lactobacillus*, they are generally considered to be beneficial bacterial genera and have been found to alleviate obesity induced by high-fat diet in mice. Previous studies have illustrated that they can attenuates progression of nonalcoholic fatty liver disease by lowering cholesterol and steatosis [21,22]. But this seems to be the opposite of what we observed, *Ligilactobacillus* and *Lactobacillus* were significantly more dominant in the gut microbiome of db/db mice. We consider that the difference NAFLD mouse models may be a key factor contributing to this result. Although both gene editing

and diet induction can establish mouse models of NAFLD, the formation mechanism and pathophysiological changes are significantly different [23,24]. This is also the limitations of our study.

In summary, our investigation has conclusively established that semaglutide effectively mitigated liver injury and the accumulation of hepatic fat in db/db mice by enhancing both glucose metabolism and lipid profiles. Furthermore, semaglutide demonstrated the capacity to enhance the gut microbiota and fostering intestinal barrier repair in db/db mice. The improvement of gut microbiota reduces intestinal inflammation and hepatic steatosis by increasing the content of intestinal short-chain fatty acids, which may provide additional ideas for the treatment of NAFLD. Whether the above microbiota can be the relevant targets for the prevention and improvement of NAFLD and the specific mechanisms still need to be further explored. Nonetheless, it is imperative to underscore that the potential connection between the pathogenesis and progression of fatty liver disease and intestinal dysbiosis demands further in-depth exploration and scrutiny.

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CRediT authorship contribution statement

Tuohua Mao: Formal analysis, Data curation. **Chenxuan Zhang:** Writing – review & editing, Writing – original draft, Software. **Shuang Yang:** Resources, Data curation. **Yingying Bi:** Investigation, Formal analysis. **Man Li:** Resources, Project administration. **Jia Yu:** Resources, Methodology.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: TUOHUA MAO reports financial support was provided by National Natural Science Foundation of China. If there are other authors, they 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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