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ORIGINAL ARTICLE

Lactobacillus murinus alleviates insulin resistance via promoting L-citrulline synthesis

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Abstract

Aims The role of *Lactobacillus murinus* as a potential probiotic is being explored. Our objectives were to explore the effects of *Lactobacillus murinus* on insulin resistance and the underlying mechanism.

Methods Insulin resistance animal models were applied to study the effect of *L. murinus* and the underlying mechanism by six weeks of treatment. Metformin was administered in vitro to analyze the growth and metabolites of *L. murinus*. Serum metabolites were further analyzed after *L. murinus* administration. The effect of L-citrulline and the underlying mechanism in alleviating insulin resistance were evaluated.

Results *L. murinus* not only reduced body weight gain and postprandial blood glucose (PBG) but improved impaired glucose tolerance (IGT) and insulin resistance. Moreover, *L. murinus* inhibited the secretion of pro-inflammatory factors (IL-1β, IL-6 and TNF-α) while promoted the secretion of anti-inflammatory factor (IL-10). Further, *L. murinus* promoted the expression of carnitine palmitoyl transferase 1 (CPT1) while inhibited phosphoenolpyruvate carboxykinase (PCK) and glucose-6-phosphatase (G6Pase). A total of 147 metabolites of *L. murinus* were identified, in which the content of L-citrulline increased to 7.94 times after metformin regulation. Further, the serum concentration of L-citrulline significantly increased after *L. murinus* administration. Similarly, L-citrulline reduced body weight gain and PBG, improved IGT and insulin resistance. Additionally, L-citrulline improved inflammation, promoted CPT1 while inhibited PCK and G6Pase.

Conclusions *L. murinus* mediated by L-citrulline alleviated insulin resistance via promoting fatty acid oxidation and inhibiting gluconeogenesis, suggesting that supplementation of *L. murinus* could be a potential therapeutic approach for type 2 diabetes related to insulin resistance.

Keywords *Lactobacillus murinus* · L-citrulline · Impaired glucose tolerance · Insulin resistance · Probiotic

Introduction

Type 2 diabetes (T2D) is a complex metabolic disease with a high incidence worldwide, of which insulin resistance is a remarkable characteristic [1]. The number of T2D patients worldwide had nearly quadrupled due to population growth and aging [2]. The results of large-scale epidemiological studies showed that the number of T2D patients exceeds 120 million in China [3, 4]. T2D could cause serious complications, such as diabetic ketoacidosis, diabetic neuropathy, etc. [5, 6]. To prevent complications, many approaches had been adopted to improve T2D, including controlling lifestyle and hypoglycemic drug intervention [7–9]. Metformin, one of the biguanides, is the first choice in the treatment of T2D. However, it failed to achieve or maintain current glycaemic goals in T2D patients with metformin in UK primary care [10], and the incidence of common gastrointestinal adverse events of metformin was approximately 38.3% [11]. The incidence of gastrointestinal adverse events of commonly used hypoglycemic drugs rosiglitazone, one of the agents of insulin sensitization, and glyburide, one of the agents of insulin secretion, both exceeded 21% [11], and the incidence of serious adverse events of dapagliflozin, one of sodium-glucose cotransporter 2 inhibitors, exceeded 34% [12]. Although a lot of research has been accomplished, effective and low adverse events treatment methods of T2D are still being explored.

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Liver is the main target organ for insulin and plays a vital role in maintaining the homeostasis of glucose and lipid metabolism. The changes in hepatic metabolism could cause obesity, T2D, and other diseases. For example, icaritin could promote fatty acid oxidation and improve insulin sensitivity, thus improving insulin resistance [13]. It was reported that the increase of hepatic gluconeogenesis led to an increase of blood glucose, which could lead to insulin resistance, leading to T2D [14]. Previous studies have shown that the inhibition of hepatic gluconeogenesis was beneficial to reduce blood glucose and improve insulin resistance [15]. Moreover, the activation of hepatic mitochondrial fatty acid oxidation was in favor of maintaining glucose homeostasis [16]. Therefore, activating fatty acid oxidation and inhibiting gluconeogenesis in hepatic tissue can improve insulin resistance.

The human gastrointestinal tract houses a vast and diverse gut microbiota that provides nutrients and intrinsic immunity. There was increasing evidence that gut microbiota fundamentally affects human health and diseases [17]. The changes in gut microbiota were related to a series of diseases, such as Alzheimer's disease and T2D [18, 19]. In our previous study, we observed that the abundance of *Lactobacillus murinus* in insulin resistance mice fed a high-fat diet (HFD) was reduced, which was increased after metformin treatment [20]. In addition, the changes in gut microbiota caused by exertional heat stroke led to cognitive impairment in mice, in which the abundance of *L. murinus* decreased significantly [18]. Studies have shown that Kaixin-san could improve cognitive impairment and increase the abundance of *L. murinus* in Alzheimer's disease model rats [21]. As a potential probiotic, *L. murinus* could reduce inflammation related to aging and improve intestinal barrier function [22, 23]. In addition, *L. murinus* reportedly alleviated intestinal ischemia/reperfusion injury by promoting the release of interleukin-10 (IL-10) [24]. However, the role and mechanism of *L. murinus* in insulin resistance remain unclear.

The purpose of the current study is to investigate the role of *L. murinus* in body weight and insulin resistance, and the underlying molecular mechanism. The results suggest that *L. murinus* reduces body weight gain and alleviates insulin resistance by promoting the synthesis of L-citrulline, in which the underlying molecular mechanism is to improve inflammation, promote fatty acid oxidation, and inhibit gluconeogenesis. The data indicate that supplementation of *L. murinus* could be a potential therapeutic approach for T2D related to insulin resistance.

Methods

Bacterial strains and growth conditions

Lactobacillus murinus frozen stocks (Testobio Co., Ltd, Ningbo, China) were added to 5 mL de Man Rogosa and Sharpe (MRS) medium (Solarbio Science & Technology Co., Ltd., Beijing, China), which were incubated at 37ºC under aerobic conditions and passaged. After 8 ~ 10 h of growth, $OD_{625} = 0.80 \sim 1.10$ of cultures was measured, at which time the colony count was 1.5×10^9 CFU/mL by 0.5 Mcfarland standard [25]. The effects of metformin at different concentrations and different incubation times on the growth of *L. murinus* were evaluated after observing the growth for 12 h. Then, the cultures of *L. murinus* were collected for the analysis of metabolites and key enzymes. In addition, 30 µL cultures of *L. murinus* were added to 3 mL MRS medium and incubated at 37ºC under aerobic conditions for 12 h, and then used for gavage of mice. Frozen stocks of *L. murinus* (in MRS medium with 50% glycerol) were prepared and stored at − 80ºC for further experiments.

Animal experiments

Six-week-old male C57BL/6J mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). After seven days of acclimatization, the mice were randomly grouped according to body weight. The control mice were fed with HFD, and the mice in the administration group were fed a HFD with administered *L. murinus* (3×10^8 CFU/d) [24] or L-citrulline (300 mg/kg/d) [26] by oral gavage for six weeks. After sacrifice, the serum, pancreas, and liver of mice were collected for subsequent analysis. All mice were housed in static cages at 20 ~ 22ºC, under a cycle with 12 h/12 h of darkness and light. Water and food were available ad libitum. The animal study protocol was approved by the Animal Ethical and Welfare Committee (approval No. MDKN-2022-009) and followed by the National Research Council's Guide for the Care and Use of Laboratory Animals.

Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT)

After six weeks of administration, OGTT and ITT were performed to evaluate the efficacy. For OGTT, the mice were fasted for 12 h and given intragastric glucose (2.0 g/kg) [27]. The mice were fasted for 6 h and intraperitoneally injected with insulin (0.50 U/kg) for ITT [28]. Their blood glucose levels were measured using tail bleeds 0, 30, 60, 90, and 120 min after the administration of glucose or insulin.

Biochemical and histopathological analysis

The serum concentrations of fasting blood glucose (FBG), total cholesterol (TC), triglyceride, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), alanine aminotransferase (ALT), and aspartate transaminase (AST) were quantified using the kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The serum concentrations of insulin, IL-1β, IL-6, IL-10, tumor necrosis factor-α (TNF-α), lipopolysaccharide (LPS) and interferon γ (IFN-γ) were quantified using ELISA kits from Jiangsu Meimian Industrial Co., Ltd (Yancheng, China). The concentrations of zonula occludens-1 (ZO-1) and occludin in intestinal tissues were analyzed by ELISA kits. The homeostasis model assessment of insulin resistance (HOMA-IR) formula was as follows: HOMA-IR = FBG (mmol/L) × insulin (mIU/L)/22.5 [29].

After sacrifice, the liver and pancreas of the mice were collected and fixed using 4% paraformaldehyde for histopathological analysis as previously reported [27].

Metabolite analysis

After liquid nitrogen inactivation, 50 µL cultures of *L. murinus* were mixed with 150 µL pre-cooled methanol acetonitrile (1/1, v/v) and extracted for 30 min, of which ketoprofen (1000 ng/mL, Solarbio) was the internal standard; the serum samples were thawed for 30 min, then 50 µL of each sample was mixed with 150 µL methanol acetonitrile solution (containing ketoprofen as the internal standard). After vortexing, the mixture was centrifuged to precipitate the protein. Then, 100 µL supernatant was used for analysis using ultra-high performance liquid chromatography-Q Exactive hybrid quadrupole-Orbitrap high-resolution accurate mass spectrometry (UHPLC-Q-Orbitrap HRMS) (Thermo Fisher Scientific, San Jose, CA, USA) equipped with electrospray ionization (ESI) in positive and negative ion modes [30]. The samples were separated by a Waters ACQUITY UPLC BEH C_{18} column (2.1 × 100 mm, 1.7 µm) with a column temperature of 40ºC. Mobile phases A and B were 0.1% formic acid aqueous solution and acetonitrile, respectively. The injection volume was 2.0 µL with an injector temperature of 4ºC, and the flow rate was set to 0.30 mL/min with an analysis time of 15 min. Data were acquired in continuum mode from *m/z* 50 to 1200. The liquid phase and MS variables are listed in Table S1.

The data acquired was preprocessed using the Waters QI software. Metabolites of *L. murinus* were identified after subtractive MRS medium. Combining univariate analysis and multivariate analysis, the differential metabolites regulated by *L. murinus* were identified by the Human Metabolome Database (HMDB, https://hmdb.ca/). Multivariate analysis included principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA), which were performed to identify discriminating metabolites between the groups [31]. Based on their variable importance in the projection (VIP) from the OPLS-DA model and *P* value between the groups, the differential metabolites (VIP > 1.0 and $P < 0.05$) were screened.

Western blotting analysis

The liver tissue was lysed for 30 min in RIPA lysis buffer containing PMSF (Solarbio) to extract protein, which was quantified using a Bradford protein assay kit (Bio-Rad, Hercules, CA, USA). After separated using 10% gel, the protein was transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). After blocked with 5% skim milk, the membranes were incubated with primary antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:20000, #10494-1-AP, Proteintech Group, Inc.), carnitine palmitoyl transferase 1 (CPT1, 1:50000, #15184-1-AP, Proteintech), phosphoenolpyruvate carboxykinase (PCK, 1:30000, #16754-1-AP, Proteintech), and glucose-6-phosphatase (G6Pase, 1:3000, #66860-1-Ig, Proteintech) overnight at 4 °C. Then, the membranes were incubated with HRP-conjugated affinipure goat anti-rabbit IgG (1:10000, #SA00001-2, Proteintech) or goat anti-mouse IgG (1:10000, #SA00001-1, Proteintech) after washed with TBST. Protein band images were developed using the Tanon 4800 multi-automatic chemiluminescence imaging analysis system (Tanon Science & Technology Co., Ltd, Shanghai, China). The scanned images were analyzed using the ImageJ software.

Statistical analysis

The data were expressed as mean ± SEM. Statistical analysis was performed using GraphPad Prism 8.3 software. The differences between the groups were compared using two-way ANOVA, Student's t-test, or Mann-Whitney test where appropriate, and *P* values < 0.05 were considered statistically significant. The specific analysis methods and sample sizes are included in the legends.

Results

L. murinus alleviates insulin resistance

With grew well within 12 h (Fig. S1A), *L. murinus* was regulated by metformin in a concentration and dose-dependent manner (Fig. S1B, C). To investigate the role of *L. murinus* in diabetes, we used HFD-fed mice as a model [29, 32]. After

six weeks of *L. murinus* administration, the average body weight of mice was significantly lower than that of mice in HFD feeding (Fig. 1A), indicating that *L. murinus* improved obesity observed in HFD-fed mice. From the results of OGTT, compared with HFD-fed mice, 30 min postprandial blood glucose (PBG) and area under the curve (AUC) of OGTT in mice with *L. murinus* administration were significantly reduced (Fig. 1B), which showed that *L. murinus* could improve impaired glucose tolerance (IGT) induced by HFD. *L. murinus* could improve insulin sensitivity since the AUC of ITT in mice administered with *L. murinus* was significantly lower than that fed with HFD (Fig. 1C). The results of HOMA-IR revealed *L. murinus* could alleviate insulin resistance (Fig. 1D). Insulin resistance is the main cause of T2D, and it could cause the damage of pancreas and liver [27]. The results of hematoxylin-eosin (H&E) staining showed that the islets of mice fed with HFD were atrophy and disorder, which improved after *L. murinus* administration (Fig. 1E). Moreover, fat deposition was observed in liver of mice fed with HFD, whereas *L. murinus* administration significantly reduced lipid droplets from the results of H&E staining and Oil Red O staining (Fig. 1E). The results showed that *L. murinus* could improve the damage of pancreas and liver tissue. Meanwhile, *L. murinus* could regulate liver function (Fig. S2A, B) and lipid metabolism (Fig. S2C-F). In addition, *L. murinus* could improve the gut barrier (Fig. S2G, H). In conclusion, the results confirmed that *L. murinus* could alleviate insulin resistance.

L. murinus improves inflammation, promotes fatty acid oxidation and inhibits gluconeogenesis

Studies have shown that HFD resulted in inflammation [27, 29]. After *L. murinus* administration, the concentration of IL-1β was significantly decreased compared with mice fed with HFD (Fig. 2A). Similarly, the concentrations of IL-6 and TNF-α in mice administered with *L. murinus* were significantly lower than HFD-mice (Fig. 2B, C). On the contrary, the concentration of anti-inflammatory factor IL-10 was significantly increased (Fig. 2D), and the concentration of cytokine IFN-γ was significantly increased (Fig. 2E) after *L. murinus* administration. The evidence indicated that *L. murinus* could improve inflammation. The results of Western blotting analysis demonstrated that the expression level of CPT1, a key rate-limiting enzyme of fatty acid oxidation, was increased in the liver of mice with *L. murinus* administration compared to HFD-fed mice. Moreover, compared with HFD-fed mice, the expression levels of PCK and G6Pase, the key rate-limiting enzymes of hepatic gluconeogenesis [15], were significantly decreased in mice administered with *L. murinus* (Fig. 2F). To sum up, these evidences showed that *L. murinus* could improve inflammation, promote fatty acid oxidation and inhibit gluconeogenesis, thus alleviating insulin resistance.

Synthesis of L-citrulline by *L. murinus* via ornithine transcarbamylase

Gut microbiota was regarded as a covert endocrine organ with a strong metabolic function, which played a crucial

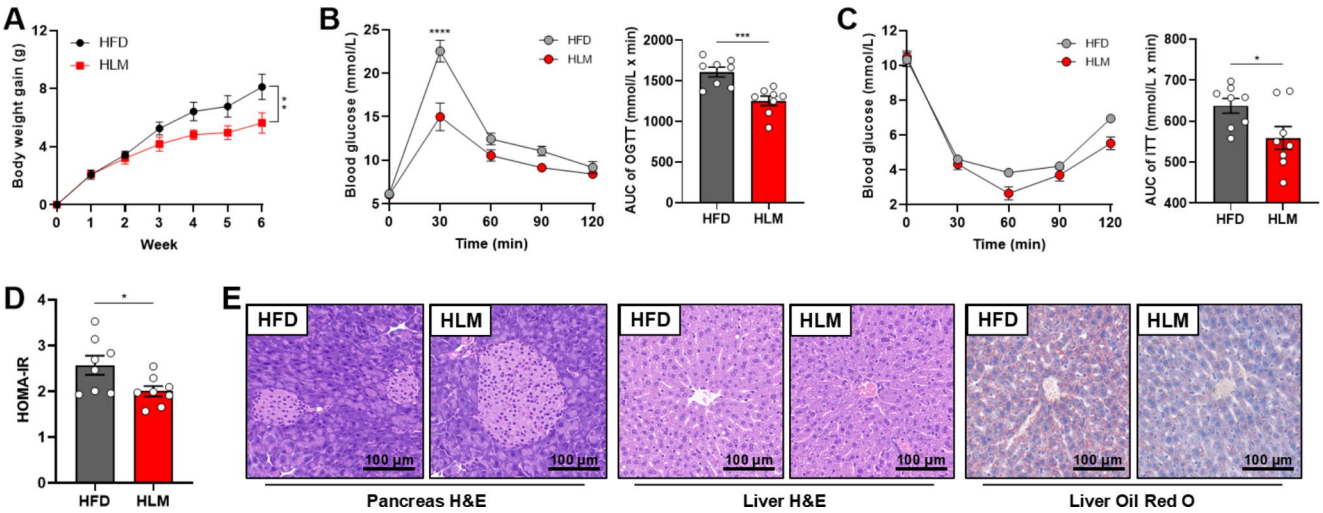

Fig. 1 *L. murinus* alleviates insulin resistance. *L. murinus* improves HFD-induced body weight gain (**A**), impaired glucose tolerance (**B**), insulin sensitivity (**C**) and insulin resistance (**D**). (**E**) Representative photomicrographs of pancreatic tissue with H&E staining, and liver tissue with H&E staining and Oil Red O staining (magnification, ×40, 100 μm). Data are means ± SEM ($n = 8$). For body weight gain, statistical analysis was performed using two-way ANOVA, and the rest of the statistics were performed with Student's t-test, $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$. HFD, high-fat diet; HLM, high-fat diet + *L. murinus*; OGTT, oral glucose tolerance test; AUC, area under the curve; ITT, insulin tolerance test; HOMA-IR, homeostasis model assessment of insulin resistance

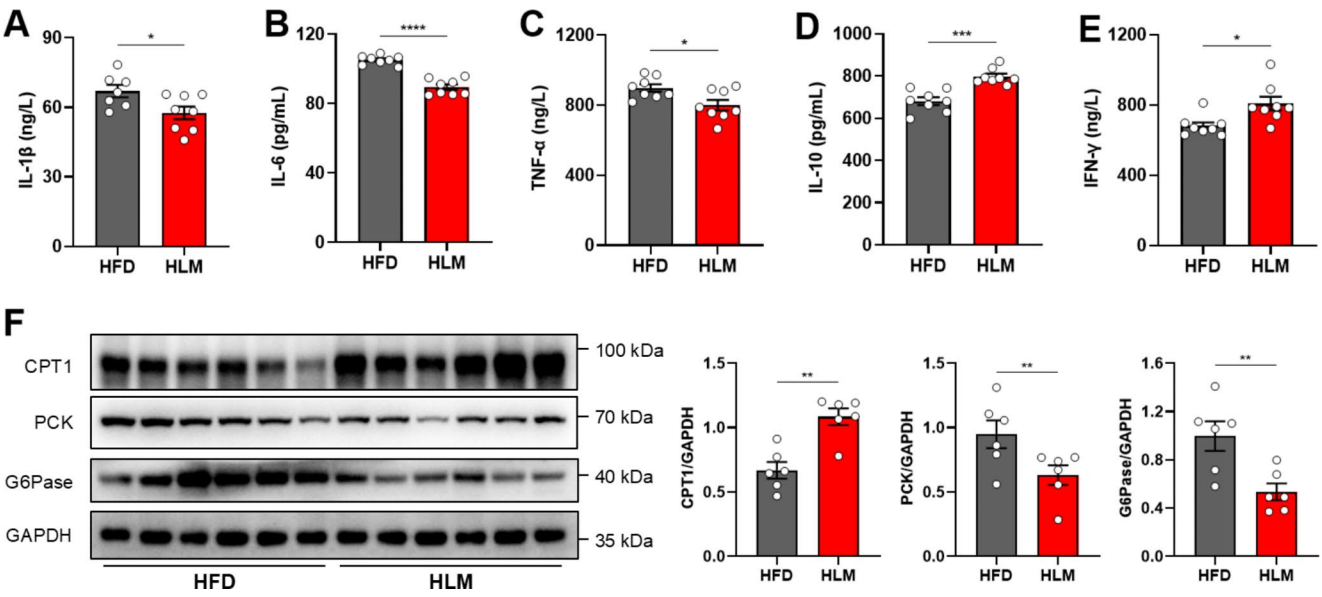

Fig. 2 *L. murinus* improves inflammation, promotes fatty acid oxidation and inhibits gluconeogenesis. *L. murinus* inhibits the secretion of IL-1β (**A**), IL-6 (**B**), and TNF-α (**C**), while promotes the secretion of IL-10 (**D**) and IFN-γ (**E**). (**F**) *L. murinus* significantly increased CPT1 expression while decreased PCK and G6Pase expression, and quantifications of CPT1, PCK and G6Pase are shown on the right. Data are means ± SEM ($n = 6/8$). For IFN-γ and CPT1, Mann-Whitney test was employed and the rest of the statistics were performed with Student's t-test, $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. HFD, high-fat diet; HLM, high-fat diet + *L. murinus*; IL-1β, interleukin 1β; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor α; IL-10, interleukin 10; IFN-γ, interferon-γ; CPT1, carnitine palmitoyl transferase 1; PCK, phosphoenolpyruvate carboxykinase; G6Pase, glucose-6-phosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

role in regulating host metabolism by producing bioactive metabolites [33, 34]. It was speculated that the metabolites produced by *L. murinus* could play important roles in alleviating insulin resistance. Hence, it was of interest to identify metabolites of *L. murinus*. Accordingly, the cultures of *L. murinus* were used for the analysis of metabolites based on UHPLC-Q-Orbitrap HRMS (Fig. S3). After subtractive MRS medium, a total of 147 metabolites of *L. murinus* were identified (Table S2), in which 70 metabolites were regulated by metformin. These regulated metabolites included amino acids, carnitine and fatty acids, etc. Surprisingly, the content of L-citrulline increased to 7.94 times after metformin regulation. As shown in Fig. 3A, N-acetylornithine was synthesized from L-glutamic acid as a precursor, and further metabolized into L-ornithine. Further, L-ornithine generated L-citrulline by ornithine transcarbamylase (OTC). L-Citrulline was metabolized to produce L-arginine by arginosuccinase (AS). The results showed that the contents of L-glutamic acid, N-acetylornithine, and L-arginine were significantly decreased after metformin regulation, while the content of L-citrulline was significantly increased after metformin regulation (Fig. 3B-E). Meanwhile, the concentration of OTC in cultures of *L. murinus* treated with metformin was significantly increased, while the concentration of AS was significantly decreased (Fig. 3F, G).

In order to evaluate the regulation of serum metabolites in mice after *L. murinus* administration, we conducted metabolomics analysis based on UHPLC-Q-Orbitrap HRMS. Interestingly, the PCA score plot showed that there were significant systematic metabolic differences between HFD-mice and mice administered with *L. murinus* (Fig. S4A, B). To explore the metabolites that led to the differences, the OPLS-DA model was established (Fig. S4C, D), suggesting apparent separations between the groups. Finally, 90 differential metabolites were identified in the serum of mice after *L. murinus* administration, mainly including amino acids, dipeptides, fatty acids, lysophospholipids, etc. (Table S3). Compared with HFD-mice, the contents of 44 serum metabolites, including oleoylcarnitine, arginyl-glutamine, linolenelaidic acid, and lysophosphatidylcholine (22:4), were significantly decreased, while the contents of 46 metabolites, including L-citrulline, lysophosphatidic acid (LysoPA) (18:2), lysophosphatidylethanolamine (LysoPE) (22:5), 2-aminooctanoic acid and 2-aminoacrylic acid, were significantly increased after the administration of *L. murinus*. Surprisingly, the content of L-citrulline increased to 4.11 times after *L. murinus* administration (Fig. 3H). Therefore, we hypothesized that L-citrulline produced by *L. murinus* via OTC increased into blood, thus alleviating insulin resistance.

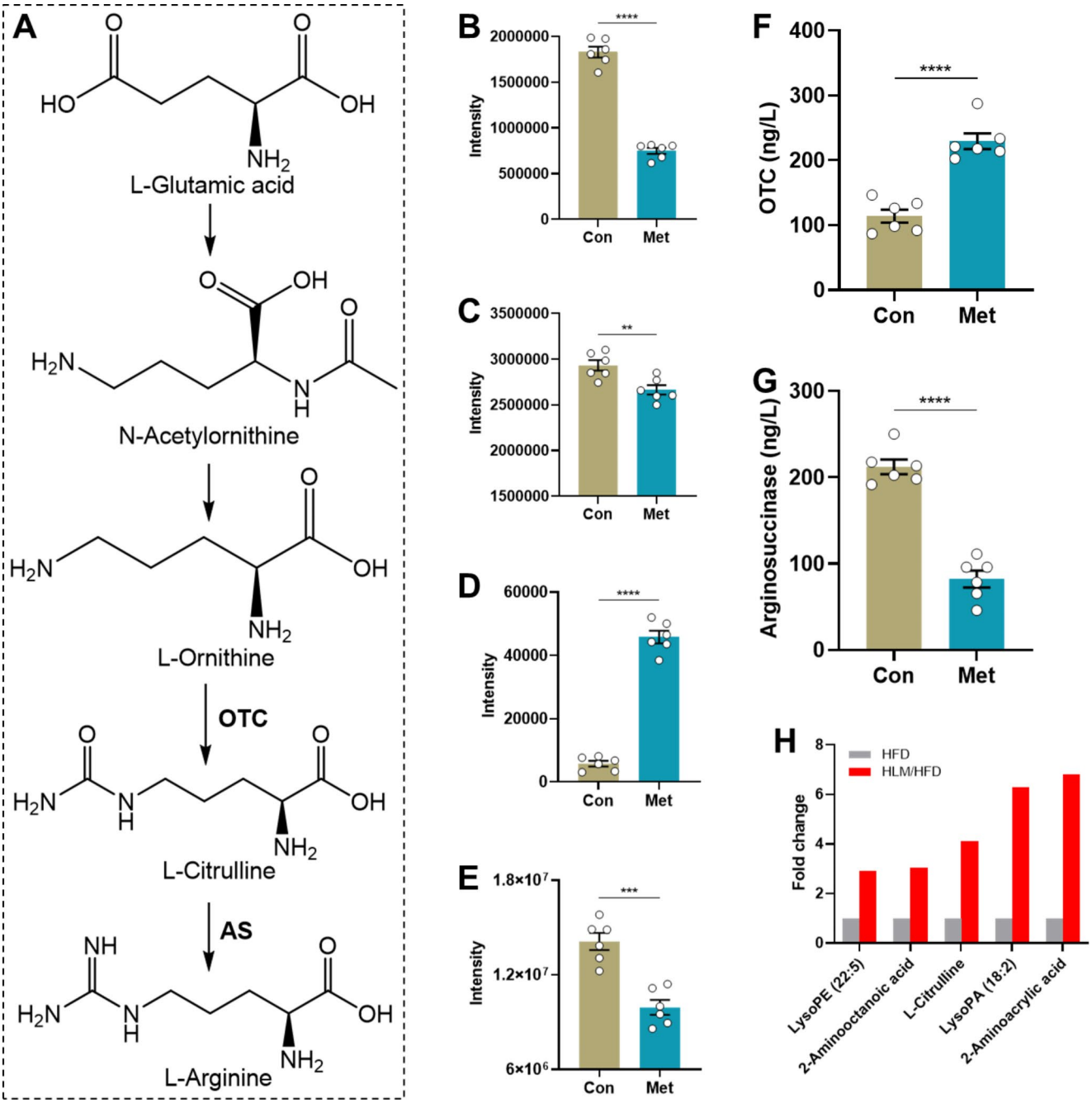

Fig. 3 The pathway of L-citrulline synthesis in *L. murinus*, and the content of related metabolites. (**A**) The pathway of L-citrulline synthesis in *L. murinus*. The contents of L-glutamic acid (**B**), N-acetylornithine (**C**), L-citrulline (**D**), and L-arginine (**E**). The concentrations of OTC (**F**) and AS (**G**) in *L. murinus* after metformin administration. (**H**) Fold change in increased TOP 5 metabolites of mice after *L. murinus* administration. Data are means ± SEM ($n = 6$). Statistical analysis was performed using Student's t-test, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. OTC, ornithine transcarbamylase; AS, arginosuccinase; Con, control; Met, metformin; HFD, high-fat diet; HLM, high-fat diet + *L. murinus*; LysoPE, lysophosphatidylethanolamine; LysoPA, lysophosphatidic acid

L-citrulline alleviates insulin resistance

In order to verify the above hypothesis, the pharmacological effect of L-citrulline on alleviating insulin resistance was evaluated. With HFD-fed mice as a model, L-citrulline was administered for 6 weeks. Obviously, L-citrulline prevented the body weight gain observed in HFD-fed mice (Fig. 4A), indicating that L-citrulline improved obesity. From the results of OGTT, compared with HFD-fed mice, the concentrations of FBG and 2 h PBG in mice administered with L-citrulline were significantly reduced, and the AUC of OGTT was also significantly reduced (Fig. 4B), indicating that L-citrulline could improve IGT induced by HFD. The AUC of ITT in mice administered with L-citrulline was significantly lower than that fed with HFD (Fig. 4C), revealing that L-citrulline could improve insulin sensitivity. Furthermore, HOMA-IR in mice administered with L-citrulline was decreased compared to HFD-fed mice (Fig. 1D), which suggested that L-citrulline could alleviate insulin resistance. From the results of H&E staining, the administration of L-citrulline improved the atrophy and disorder of islets in mice fed with HFD (Fig. 4E). Besides, from the results of H&E staining and Oil Red O staining, the administration of L-citrulline improved the fat deposition in liver of mice fed with HFD (Fig. 4E). The results suggested that L-citrulline improved the damage of pancreas and liver tissue, even though L-citrulline had no regulating effect on liver function (Fig. S5A, B). In addition, L-citrulline reduced the concentration of total cholesterol (Fig. S5C-F). In conclusion, the results confirmed that L-citrulline could alleviate insulin resistance.

L-citrulline improves inflammation, promotes fatty acid oxidation and inhibits gluconeogenesis

Compared with mice fed with HFD, the concentration of IL-1β was significantly decreased after L-citrulline administration (Fig. 5A). Analogously, the concentrations of IL-6 and TNF-α in mice administered with L-citrulline were significantly lower than that fed with HFD (Fig. 5B, C). LPS, a major component of the outer membrane of Gram-negative bacteria, caused inflammation by activating the expression of the inflammatory cytokine gene [35]. The serum concentration of LPS in mice administered with L-citrulline was significantly lower than that fed with HFD (Fig. 5D). Contrary, compared to the mice fed with HFD, the concentrations of anti-inflammatory factor IL-10 and cytokine IFN-γ were significantly increased after L-citrulline administration (Fig. 5E, F). Additionally, compared with mice fed with HFD, the expression level of CPT1 was increased, while the expression levels of PCK and G6Pase were decreased in the liver of mice with L-citrulline administration (Fig. 5G). In short, these evidences showed that L-citrulline could improve inflammation, promote fatty acid oxidation and inhibit gluconeogenesis, thus alleviating insulin resistance.

Discussion

T2D has a high incidence worldwide. To prevent complications, hypoglycemic drug intervention is the main treatment of T2D, including metformin, rosiglitazone, glyburide, dapagliflozin, and so on [10–12]. However, there are high incidence of adverse events with these commonly used

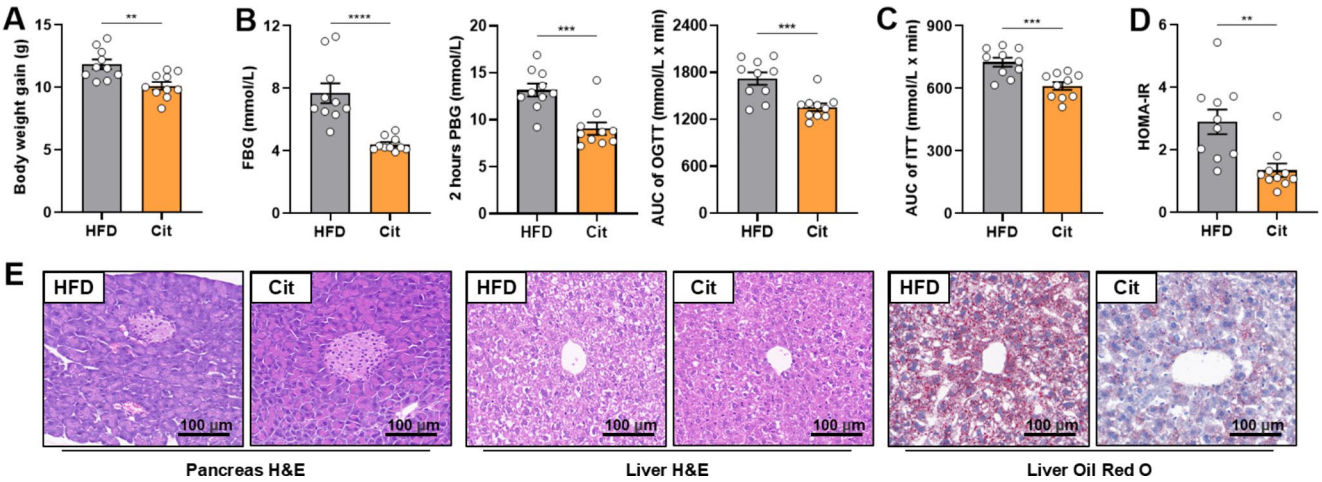

Fig. 4 L-citrulline alleviates insulin resistance. L-citrulline improves HFD-induced body weight gain (**A**), impaired glucose tolerance (**B**), insulin sensitivity (**C**) and insulin resistance (**D**). (**E**) Representative photomicrographs of pancreatic tissue with H&E staining, and liver tissue with H&E staining and Oil Red O staining (magnification, ×40, 100 μm). Data are means ± SEM ($n = 10$). For 2 h PBG and HOMA-IR, Mann-Whitney test was employed and the rest of the statistics were performed with Student's t-test, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. HFD, high-fat diet; Cit, L-citrulline; FBG, fasting blood glucose; PBG, postprandial blood glucose; OGTT, oral glucose tolerance test; AUC, area under the curve; ITT, insulin tolerance test; HOMA-IR, homeostasis model assessment of insulin resistance

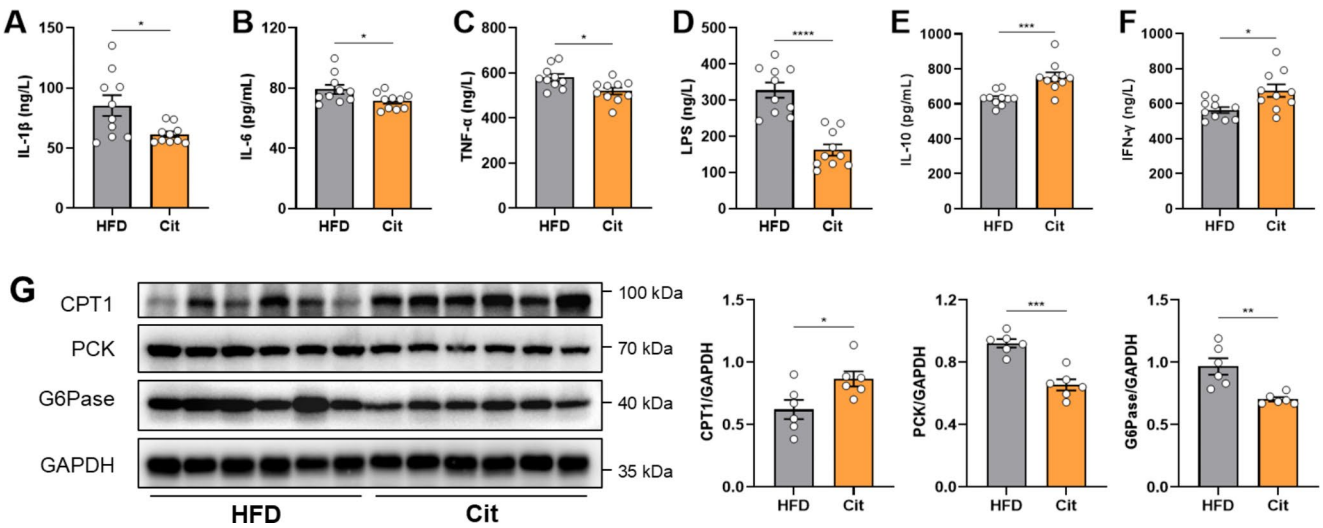

Fig. 5 L-citrulline improves inflammation, promotes fatty acid oxidation and inhibits gluconeogenesis. L-Citrulline inhibits the secretion of IL-1β (**A**), IL-6 (**B**) TNF-α (**C**), and LPS (**D**), while promotes the secretion of IL-10 (**E**) and IFN-γ (**F**). (**G**) L-citrulline significantly increased CPT1 expression while decreased PCK and G6Pase expression, and quantifications of CPT1, PCK and G6Pase are shown on the right. Data are means ± SEM ($n = 6/10$). For IFN-γ and CPT1, Mann-Whitney test was employed and the rest of the statistics were performed with Student's t-test, $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. HFD, high-fat diet; Cit, L-citrulline; IL-1β, interleukin 1β; TNF-α, tumor necrosis factor α; LPS, lipopolysaccharide; IL-10, interleukin 10; IFN-γ, interferon-γ; CPT1, carnitine palmitoyl transferase 1; PCK, phosphoenolpyruvate carboxykinase; G6Pase, glucose-6-phosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

hypoglycemic agents. Biological agent is a new type of drug for treating T2D, which has the advantages of excellent therapeutic effect, low adverse reactions, and high drug stability. Probiotics, as a natural and safe microbial agent, have gradually entered our research field of vision. As a potential probiotic, the role and mechanism of *L. murinus* in insulin resistance remain unclear.

In the present study, for the first time, we confirmed that *L. murinus* supplementation effectively alleviated insulin resistance. In addition, *L. murinus* improved inflammation by promoting the secretion of anti-inflammatory factors and inhibiting the secretion of pro-inflammatory factors. The results in vitro and in vivo suggested that *L. murinus* promoted the synthesis of L-citrulline. Furthermore, L-citrulline alleviated insulin resistance. Congruously, L-citrulline improved inflammation by promoting the secretion of anti-inflammatory factors and inhibiting the secretion of pro-inflammatory factors. It suggested that the anti-diabetic effect of *L. murinus* was mediated by L-citrulline. It was well known that long-term medication was necessary for patients with T2D. However, prolonged medication could reduce curative effect [10]. It is shocking that adverse events were of frequent occurrence. Therefore, we try to explore a potential therapeutic approach to alleviate insulin resistance from the perspective of probiotics.

Gut microbiota is a complex ecosystem susceptible to the surrounding environment and diet [18]. We previously confirmed that insulin resistance induced significant gut microbiota disorders and indicated that gut microbial metabolites play an important regulatory role in insulin resistance [20, 27]. Previous experiments showed that depletion of gut commensal bacteria could attenuate intestinal inflammation and cognitive dysfunction [21, 24], of which the abundance of *L. murinus* decreased. However, the extensive diversity of the gut microbiome hindered the precise determination of the role of *L. murinus* in insulin resistance. For the first time, herein, we confirmed that *L. murinus* alleviated insulin resistance. Lactobacillus murinus strains have previously been isolated and identified from humans and animals [36]. Few reports have indicated the application of *L. murinus* in host health and disease. It has been shown that It has been shown that *L. murinus* might be used as a potential probiotic to reduce the incidence of delayed sepsis in neonates [37]. In addition, another study showed that *L. murinus* alleviated intestinal ischemia/reperfusion injury through promoting the release of IL-10 from M2 macrophages [24]. Various *L. murinus* strains have been further characterized as potential probiotics in the food formulation industry. With increased public interest in *L. murinus*-containing probiotics, the impacts of *L. murinus* on insulin resistance are beginning to be unraveled.

Gut microbiota exerts a protective role for the gut barrier under steady-state conditions due to its high level of plasticity. In the current study, the results indicated that *L. murinus* could promote the expression of ZO-1 and occludin (Fig. S2G, H), which are important proteins for maintaining the gut barrier [38]. The dysfunction of the gut barrier might cause nutrient absorption disorders. It hence promoted islet

β cells to secrete more insulin to maintain blood glucose, which could reduce insulin sensitivity, thus aggravating insulin resistance and the occurrence and development of T2D [39, 40]. However, there was no evidence that *L. murinus* could alleviate insulin resistance. In the current study, *L. murinus* not only improved the gut barrier, but also reduced 30 min PBG, improved IGT, insulin sensitivity, and insulin resistance (Fig. 1B-D). Furthermore, *L. murinus* regulated the secretion of cytokines, resulting in the decrease of pro-inflammatory factors and the increase of anti-inflammatory factors, thus improving inflammation, which was consistent with previous studies [24, 41]. Studies have shown that the increase of IL-6 secretion in brown adipocytes could lead to the failure to normally regulate glucose metabolism and decompose fat, thus causing obesity [42, 43], which might be one of the reasons why *L. murinus* reduced body weight.

In order to confirm the hypothesis that the effect of *L. murinus* on alleviating insulin resistance might be mediated by its secondary metabolites, we analyzed the metabolites of *L. murinus* and serum of the mice. The results indicated that L-citrulline, a metabolite of *L. murinus*, increased while the contents of L-glutamic acid, N-acetylornithine, and L-arginine decreased after metformin regulation (Fig. 3B-E), which were in the pathway of L-citrulline synthesis and metabolism (Fig. 3A). Interestingly, after metformin regulation, the content of L-citrulline increased to 7.94 times, and the concentration of OTC, an enzyme for synthesizing L-citrulline, increased while the concentration of AS, an enzyme for metabolizing L-citrulline, decreased (Fig. 3F, G). Further, the serum concentration of L-citrulline significantly increased after *L. murinus* administration. In our previous investigation of T2D-related metabolomics, L-citrulline was decreased in the serum and plasma of patients [44]. Thus, L-citrulline caught our attention.

Clinical evidence showed that L-citrulline had an anti-diabetic effect by improving insulin sensitivity, and reducing blood glucose as well as pro-inflammatory factors [26, 45, 46]. With HFD-fed mice as a model, the results suggested that L-citrulline reduced FBG, 2 h PBG, insulin resistance and pro-inflammatory factors, while increased insulin sensitivity and anti-inflammatory factors (Figs. 4B-D and 5A-F). The potential mechanism was further explored. Correspondingly, the results showed that L-citrulline inhibited the expression levels of PCK and G6Pase, indicating that L-citrulline inhibited gluconeogenesis, which was consistent with the results of OGTT. Additionally, L-citrulline promoted the expression level of CPT1, indicating L-citrulline promoted fatty acid oxidation, which was consistent with the results of body weight. The above results were also confirmed after *L. murinus* administration.

Conclusions

L. murinus reduced body weight and alleviated insulin resistance. The effect of *L. murinus* was mediated by promoting the synthesis of L-citrulline, which improved inflammation, promoted fatty acid oxidation and inhibited gluconeogenesis, thus reducing body weight and alleviating insulin resistance. Our study provides novel evidence for understanding the effect of *L. murinus* on body weight and insulin resistance, contributing to the search for a therapeutic strategy in the treatment of T2D from the perspective of probiotics.

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Author contributions All authors participated in the writing and revision of the manuscript and approved this manuscript to be submitted for publication. JL and DY contributed to the design and conception of the manuscript. JL and ZS conducted the statistical analysis. JL, ZS, ZM, and LD performed experiments. DY was the guarantor who accepted full responsibility for the work and/or the conduct of the study, had access to the data, and controlled the decision to publish.

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Data availability Data are available upon request.

Declarations

Conflict of interest All authors have no conflict of interest.

Ethical approval The study was approved by the Animal Ethical and Welfare Committee (approval No. MDKN-2022-009).

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