The Metabolite β -Hydroxybutyrate of *Lactobacillus Plantarum* YZX21 Improves Type 2 Diabetes By Promoting Intestinal Secretion of GLP-1

Zhe Zhang

Ocean University of China

Xi Liang

Ocean University of China

Lingjun Tong

Ocean University of China

Youyou Lv

Ocean University of China

Haiyan Lu

Ocean University of China

Maozhen Zhao

Ocean University of China

Pimin Gong

Ocean University of China

Tongjie Liu

Ocean University of China

Huaxi Yi

Ocean University of China

Lan Wei Zhang (✉ zhanglanwei@ouc.edu.cn)

Ocean University of China <https://orcid.org/0000-0003-3459-2975>

Research

Keywords: Probiotics, Glucose Homeostasis, Metabolomics, GLP-1 Secretion, Insulin Resistance

Posted Date: September 16th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-850464/v1>

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Abstract

Background: Probiotics and their metabolites regulate type 2 diabetes mellitus (T2DM) by promoting GLP-1 secretion, but the mechanism has not yet been fully clarified.

Results: In order to reveal the effect of *Lactobacillus plantarum* YZX21 on the regulation of T2DM, type 2 diabetic C57BL/6 mice induced by a high-fat diet and streptozotocin (STZ) were divided into different groups and were daily treated with *L. plantarum* YZX21 for 8 weeks. We identified that *L. plantarum* YZX21 reduced blood glucose, insulin levels and HOME-IR. Histopathology was found that *L. plantarum* YZX21 restored the mouse islet cells morphology and increased insulin secretion. The Elisa and immunohistochemical staining revealed concentration of glucagon-like peptide-1 (GLP-1) was increased in the mice colon. UPLC-MS/MS based widely-targeted metabolomics analysis was used to identify the differential intestinal metabolites in the mice colon. It was found that the metabolite β -Hydroxybutyrate (BHB) of *L. plantarum* YZX21 had a negative association with T2DM. Subsequently, type 2 diabetic C57BL/6 mice were established and used to verify hyperglycemic effect by daily treatment with BHB for 8 weeks. It was found that BHB improved pathologicemia, insulin resistance and increased the intestinal GLP-1 levels, especially reduced free fatty acid (FFA) levels. The expression of G protein-coupled receptor (GPCR) was verified by RT-PCR. The GPR109a receptor was significantly stimulated which up-regulated the expression of GLP-1, but not GPR41 and GPR43 in the mice colon.

Conclusions: We found that the hypoglycemic effect of *L. plantarum* YZX21 was demonstrated by increasing the intestinal GLP-1 levels in the diabetic mice. Through the widely-targeted metabolomics, we identified the close correlation between serum concentrations of BHB and T2DM. In T2DM mice model, BHB reduced the FFA levels and increased the intestinal GLP-1 levels, which is associated with the downregulated expression of GPR109a. These results demonstrated that *L. plantarum* YZX21 alleviated T2DM by upregulating BHB/GPR109a/GLP-1 associated pathway.

Background

Type 2 diabetes mellitus (T2DM) was the third independent risk factor for premature mortality and disability in individuals [1]. Previous studies showed that hormone glucagon-like peptide-1 (GLP-1) secreted by intestinal L cells stimulated insulin secretion and controlled blood glucose stability [2]. Therefore, increasing the production and secretion of endogenous GLP-1 had been widely concerned [3].

It is reported that probiotics can colonize in the intestine to regulate blood glucose by increasing the secretion of GLP-1 [4, 5]. The probiotics regulated the expression of GLP-1 through metabolites such as bile acids, short chain fatty acids and tryptophan derivatives [6]. However, there was no direct correlation between these metabolites and FFA concentrations. It was reported that prolonged FFA stimulation or long-term high-fat diet led to impaired secretion of GLP-1 [7]. Since more than half of insulin secretion after eating is induced by GLP-1, impaired GLP-1 secretion may be an important mechanism of lipotoxicity

causing blood glucose disorders [8, 9]. However, the mechanism which probiotics regulated FFA to increasing secretion of GLP-1 was not fully understood.

Metabolomics has been widely used in research related to disease diagnosis, as well as the exploration of drug action mechanisms and new biomarkers [10]. Widely-targeted metabolomics can reflect the metabolic pathways and be commonly used in research on the response mechanisms of different diseases and microorganisms [11]. With the help of Metabolomics, it was expected to reveal the mechanism of probiotics regulating GLP-1.

This paper used T2DM mice model to study the effects of *Lactobacillus plantarum* (*L.plantarum*) YZX21 on the glucose homeostasis as well as the secretion of intestinal GLP-1. And through the widely-targeted metabolomic analyses was to identify the target metabolites that stimulate GLP-1 secretion and reveal the regulatory mechanism of probiotics.

Results

The effect of *L.plantarum* YZX21 on body weight, food intake and blood glucose of mice

From the fifth week, diabetic mice showed typical symptoms of diabetes. The body weight and food intake of mice results are shown in Fig. 1A and 1B. Compared with control group, all diabetic mice showed significant weight loss from fifth week to twelfth week ($p < 0.05$). After supplementing with *L.plantarum* YZX21, the weight of diabetic mice recovered significantly from 20.34g to 24.24g, which was not significantly different from the M group. The food intake of diabetic mice increased significantly from fifth week to twelfth week. After the supplement of *L.plantarum* YZX21, the diet of mice was relieved from 13.28g to 7.03g ($p < 0.05$). The FBG and PBG levels are shown in Fig. 1C and 1D. Compared with control group, the FBG levels of diabetic mice were higher than 7.0 mmol/L and the PBG levels were higher than 11.1mmol/L. The FBG of mice in the *L.plantarum* YZX21 group decreased from 12.26 mmol/L to 7.13 mmol/L ($p < 0.05$). The PBG levels was significantly reduced, from 24.68 mmol/L to 12.95 mmol/L ($p < 0.05$). The OGTT results are shown in Fig. 1E. Compared with control group, the curve area was almost the 3 times larger than the control group. In the *L.plantarum* YZX21 supplement group, The area under the blood OGTT curve was significantly reduced, which was no different from the M group (Fig. 1F).

The effects of *L.plantarum* YZX21 on insulin secretion and pancreatic islets

The results of insulin intraperitoneal injection (ITT) are shown in Fig. 2A. Compared with control group, the area of AUCglucose was 3 times than the control group. The insulin sensitivity of mice in the *L.plantarum* YZX21 group was significantly improved, and the area of AUCglucose decreased significantly (Fig. 2B), down 2.4 times ($p < 0.05$). Figure 2C and D show the fasting insulin levels and HOMA-IR. Compared with the control group, the fasting insulin levels of diabetic mice were significantly higher, and the HOMA-IR were also at high levels. After *L.plantarum* YZX21 intervention, serum insulin levels were significantly reduced from 38.42 mmol/L to 24.14 mmol/L and the HOMA-IR was decreased from 17.60 to 7.65, which was no different from the M group. The effects of *L.plantarum* YZX21 on islet

cells was further verified by histopathology. The histopathological staining results of the pancreas are shown in Fig. 2E. The pancreatic islet cells were round or oval, with clear cells, rich and uniform cytoplasm in control group. In diabetic mice, the area ratio of islets to pancreas decreased significantly, the cells showed irregular structures, and the volume of islet cells decreased. In the *L.plantarum* YZX21 group, some pancreatic islet cells shrank and stained very lightly, but the cell morphology was complete, and the morphology was close to normal pancreas. The results of immunofluorescence staining showed (Fig. 2F) that the insulin of the control group occupied most of the pancreatic islet area, and insulin secretion was normal. While the insulin signal of diabetic mice was greatly reduced and the glucagon signal was significantly increased. In the *L.plantarum* YZX21 group, insulin secretion was significantly increased, glucagon secretion was decreased, and the release pressure of islet hormone was significantly relieved.

The colonize site of *L.plantarum* YZX21 and its effects on the intestinal GLP-1 secretion

Through the fluorescence staining experiment (Fig. 3A), it was found that *L.plantarum* YZX21 gavaged for 6 h showed high fluorescence intensity in the colon, and at 12 h, the colon still maintained high fluorescence intensity. This suggested that *L.plantarum* YZX21 was likely to mainly colonize the colon. As shown in Fig. 3B, the number of GLP-1 positive cells in the colonic mucosa of diabetic mice decreased. While after supplementation with *L.plantarum* YZX21, the colonic GLP-1 positive cells increased significantly from 17 to 31 (Fig. 3C). Consistent with this finding, in diabetic mice, the content of GLP-1 in the colon was significantly reduced (Fig. 3D). After supplementing with *L.plantarum* YZX21, the content of GLP-1 was significantly increased from 4.23 pmol/L to 11.07 pmol/L. Further the GLP-1 regulator gene GCG and PC3 mRNA levels were revealed in Fig. 3E and F. It was found that the expressions of GCG and PC3 were significantly increased in the colon after supplement of *L.plantarum* YZX21, where the expression was increased both about 3 times.

Metabonomics analysis of intestinal metabolites of *L.plantarum* YZX21

A significant difference between the metabolites of the diabetes group and the *L.plantarum* YZX21 group was observed in the OPLS-DA score chart (Fig. 4A), which suggested that there was a significant difference between the intestinal metabolism profiles of the two groups. Based on the variable importance in the projection value (VIP) greater than 1, the fold change value than 2 and *p*-value less than 0.05, it revealed that there were 87 metabolites that are significantly different between the diabetes group and the *L.plantarum* YZX21 intervention group (Fig. 4B). Based on the VIP score and the fold change value, we listed the top 10 metabolites in Table S3. FFA was the most changed metabolite, which was significantly reduced in YZX21 group. Through human metabolomic database (HMDB) and association analysis of metabolites with type 2 diabetes (Fig. 4C), it is found that BHB was closely related to FFA metabolic pathway and T2DM. It was found that free fatty acids (FFA) were highly increased in D group, which was nearly 4 times that of the YZX21 group (Fig. 5D). And BHB of *L.plantum* YZX21 group increased significantly, which was more than 3.5 times higher than that of D group (Fig. 5E).

Effects of BHB supplement on blood glucose and pancreatic islets in type 2 diabetic mice

To further characterize the effects of BHB on T2DM and FFA, we measured effects of BHB supplement on diabetes-related indicators through the diabetic mice. The FBG levels of mice are shown in Fig. 5A. Compared with control group, the FBG levels of diabetic mice were higher than 7.0 mmol/L. The FBG of mice in the BHB group decreased from 9.72mmol/L to 7.52mmol/L ($p < 0.05$). The PBG levels were shown in Fig. 5B. The PBG levels of diabetic mice were higher than 11.1mmol/L. After supplementing BHB, the PBG decreased significantly, from 21.15mmol/L to 11.88mmol/L ($p < 0.05$). Fasting insulin levels and HOMA-IR are shown in Fig. 5C and D. Compared with the control group, the fasting insulin levels of diabetic mice were significantly higher, and the HOMA-IR levels were also at high levels. However BHB intervention significantly reduced the serum insulin levels from 37.67 mIU/L to 21.19 mIU/L and HOMA-IR from 16.57 to 7.32, which were no difference from the M group. The histopathology was shown in Fig. 5E. The islet cells in the control group were rich and uniform. In diabetic mice, the cells presented irregular cell structure. In the BHB group, except for occasional lymphocytes and hemorrhage, the morphology and structure of the islet cells were similar to normal pancreas. Immunofluorescence staining results (Fig. 5F) the insulin signal of diabetic mice was significantly decreased and glucagon signal was significantly increased. In the BHB treatment group, insulin secretion was significantly increased, glucagon secretion was decreased.

Effects of BHB on intestinal GLP-1 hormone

The number of GLP-1 positive cells in the colonic mucosa of diabetic mice was reduced. While the supplementation of BHB, the positive cells was restored to the level in control group (Fig. 6A and B). Intestinal GLP-1 levels were significantly reduced in diabetic mice (Fig. 6C) and increased significantly after BHB supplementation, from 3.98 to 7.61 pmol/L.

Effect of BHB on FFA and TG levels

The experimental results showed that the diabetic mice showed significant FFA metabolic disorder, and the serum FFA level was significantly increased, which was 2.73-fold that of the control group (Fig. 7A). Serum TG level was also significantly increased, 2.41 times that of control mice (Fig. 7B). After BHB intervention, FFA disorder of diabetic mice were significantly improved, FFA level decreased to 187.56 μ mol/L, significantly lower than the diabetic group. And TG level decreased to 1.56 mmol/L, also lower than the diabetic group.

Effect of BHB on GPR109a, GPR41 and GPR43 in colon

In order to further explain the way BHB regulates FFA, fluorescence immunoassay was used to detect the expression of GPR109a in colon. The results in Fig. 8A showed that the expression of GPR109a in the colon of the diabetic mice were significantly reduced. After BHB intervention, its levels were significantly restored, and the fluorescence intensity was increased, and basically returned to a high level. GPR41 and GPR43 which highly expressed in colon tissue were also possibly receptors of BHB. Further testing the expression of other GPRs in the colon (Fig. 8B), it found that the diabetic group has a significant inhibiting effect on GPR41, GPR43 and GPR109a. After supplementation with BHB, it had a significant up-

regulation effect on the expression of GPR109a, while the influence of GPR41 and GPR43 was not obvious.

Discussion

Increased appetite and weight loss were the characteristics of T2DM [12]. After supplementation with *L.plantarum* YZX21, the weight of diabetic mice was restored, while food intake decreased. It was suggested that the glucose utilization was increased and glycogen and fat conversion was reduced. FBG and PBG were the criteria for diagnosing diabetes [13]. After supplementing *L.plantarum* YZX21, FBG and PBG were all relieved, and basically returned to normal levels. OGTT and its AUCglucose were the indicators of ability of pancreatic β -cells to regulate blood glucose [14]. The blood glucose of diabetic mice climbed rapidly and decreased slowly. The area of AUCglucose of diabetes was significantly higher. However supplementing *L.plantarum* YZX21, the peak blood glucose level decreased, and the blood glucose level dropped smoothly. This suggested that after supplementing with *L.plantarum* YZX21, the ability of pancreatic β -cells to regulate blood glucose was significantly enhanced.

Insulin is the only hypoglycemic hormone secreted by β cells. The main reason for the increase blood glucose in T2DM is insulin parasecretion [15]. The ITT experiment reflected the sensitivity of insulin in body [16]. After supplementing *L.plantarum* YZX21, the insulin sensitivity of the mice was obviously restored, and the area of AUCglucose was reduced. Fasting insulin level and HOME-IR are other key indicators of the body's sensitivity to insulin [17]. After supplementing *L.plantarum* YZX21, fasting insulin levels and HOME-IR were adjusted. These results suggested that *L.plantarum* YZX21 improved insulin sensitivity in T2DM mice. Histopathology also found the *L.plantarum* YZX21 supplement group reduced insulin resistance, protected the morphology of islet cells, restored insulin secretion, and inhibited insulin secretion. These results suggested that *L.plantarum* YZX21 reduced blood glucose levels in mice by improving insulin secretion. Improving insulin secretion and insulin sensitivity were the key of probiotics to play the role of hypoglycemia.

Through the fluorescence colonization experiment, we found that *L.plantarum* YZX21 reached the colon and maintained with a high intensity. This suggested that *L.plantarum* YZX21 was likely to colonize in colon, which were a large number of L cells. GLP-1 is the intestinal hormone that protects the morphology of pancreatic islets, increase insulin secretion, and reduce foreign insulin resistance [18]. We also found that the number of GLP-1 positive cells and the GLP-1 content increased significantly in the colon. This suggested that *L.plantarum* YZX21 was likely to increase and regulate the content of GLP-1 by colonizing the colon.

Intestinal metabolites are the key to the connection between strains and intestinal cells [19]. The increase or decrease of specific metabolites will affect the metabolism of the host [20, 21]. Specific metabolites are likely to be key components of hypoglycemic function. Through metabolomics techniques, we found that the FFA content in the intestine was significantly reduced in the *L.plantarum* YZX21 group. FFA has lipotoxic effects and is an important risk factor for diabetes[22]. As a kind of ketone body metabolite,

BHB was closely related to metabolic diseases. In the *L.plantarum* YZX21 group, the BHB level increased significantly. The HMDB and correlation analysis suggested a close relationship between BHB and diabetes. However, the relationship between BHB, FFA and GLP-1 was not fully understood.

BHB was a common metabolite and the most important member of the ketone body family [23]. BHB was closely related to major diseases such as energy metabolism disorders in the organism and diabetes [24]. The relationship between BHB and GLP-1 had not been studied. In particular, whether probiotics could improve GLP-1 through BHB was still unclear. We constructed T2DM mice and gavaged BHB to detect the mechanism and pathway of blood glucose regulation. After intragastric administration of BHB, the FBG and PBG of diabetic mice was decreased significantly and OGTT was improved. In the same way, insulin levels and HOME-IR in diabetic mice was also significantly improved. These results indicated that BHB intervention in type 2 diabetic mice reduced blood glucose by improving insulin resistance, which undoubtedly affects the process of T2DM. In addition, the results from pancreas histopathology and immunofluorescence analysis suggested that BHB contributes to increasing the insulin secretion, protecting islet cell morphology and inhibiting apoptosis. Hypoglycemic effects of BHB came down to increase the intestinal GLP-1 secretion. It was worth noting that BHB reduced TG and FFA levels, respectively. Metabolomics prompted that FFA was the greatest decrease in intestinal contents in diabetic mice. These results again suggested that BHB was likely to change intestinal GLP-1 content by influencing FFA content.

Circulating FFA is believed to play a causal role in the pathogenesis of liver and peripheral insulin resistance [25, 26] and is also a hallmark of T2DM [27]. Too much FFA aggravates insulin resistance, and is called lipotoxicity [28, 29]. Studies found that high levels of FFA inhibit GLP-1 [30]. Inhibiting FFA levels can increase GLP-1 secretion, thereby improving T2DM [7, 31]. Studies have shown that BHB significantly improved tissue damage, metabolic disorders and protein metabolism [32]. As an endogenous signaling molecule, BHB binds to and activates the intestinal hydroxyl carboxylic acid receptor (HCA2) [33, 34], also known as GPR109a. BHB reduces the levels of FFAs and triglycerides in the circulation [35]. Our experiments found that after BHB intervention, serum FFA and TG levels were significantly reduced, while GLP-1 levels were significantly increased. The expression levels of its possible receptors GPR109a, GPR41 and GPR43 showed that GPR109a was mainly activated, but the effect on GPR41 and GPR43 was not obvious. Fluorescence histochemical experiments also found that BHB significantly increased the expression of GPR109a in the intestine. GPR109a in the intestine could inhibit the hydrolysis of TG into FFA and reduce its inhibitory effect on GLP-1 [36, 37]. Thereby BHB restored the secretion of GLP-1 by reducing the inhibition of FFA through activating GPR109a receptor.

Conclusions

In summary, through the widely-targeted metabolomic analyses of intestinal contents, we found that *L.plantarum* YZX21 increased the content of BHB in the intestine. As the endogenous ligand of GPR109a, BHB inhibited fat degradation and reduced FFA levels, thereby increasing GLP-1 secretion and regulating

diabetes. These results provide a new theoretical basis for the use of *L.plantarum* YZX21 to intervene in T2DM.

Materials And Methods

Experimental strains

The *L.plantarum* YZX21 was stored in the Functional Dairy and Probiotic Engineering Laboratory of Ocean University of China (preservation number CCTCC M 2020829). The strain was activated in de Man, Rogosa and Sharpe (MRS) broth at 37 °C for 48 h for twice, then inoculated in MRS broth at an inoculum size of 2% (v/v) and incubated at 37°C for 24 h. Following that, the bacteria were centrifuged at 4 °C and 5000 ×g for 5 min. Sediments washed twice with sterile PBS buffer and resuspended in PBS to a final concentration of 1×10^8 CFU/mL, stored at 4 °C before use.

Animal experiment

Experimental animals and model construction

6-week-old SPF C57BL/6J male mice (weighing 18-20g, n=80) were provided by Beijing Vital River Laboratory Animal Technology Co. Ltd. The mice were fed in a single cage with normal or high-fat feed. The specific ingredients are shown in Table S1. The animal room had constant temperature and humidity (temperature 21-25°C, humidity 40%-60%, light from 8:00-20:00 for 12 h).

Before the experiment, the mice were adaptively fed in the animal room for 1 week. The control mice (n=8) was fed with normal diet and diabetic mice (n=24) was fed with high-fat diet for 4 weeks. After 4 weeks of dietary manipulation, the control mice were treated with 50 mmol/L citrate buffer (pH 4.5) by intraperitoneal injection. And diabetic mice were given intraperitoneal injection of Streptozotocin (STZ) at a dose of 50 mg/ (kg bw) [38]. After 7 days of STZ injection, the blood glucose of the mice was measured. The fasting blood glucose of the mice ≥ 7.0 mmol/L or the PBG level ≥ 11.1 mmol/L was considered as a successful model of diabetes[13].

Animal grouping and handling

L.plantarum YZX21 supplementing experiment of mice by gavage

Control group (C group): normal mice, gavage 0.25 ml 3% PBS solution; diabetic group (D group): diabetic mice, gavage 0.25 ml 3% PBS solution; Metformin group (M group): diabetic mice, gavage 0.25 ml of 10 mg/kg.bw metformin; *L.plantarum* YZX21 group (YZX21 group): diabetic mice, gavage 1×10^8 CFU/mL.bw. The mice were given intragastric administration once daily from weeks 1 to 12.

β -Hydroxybutyrate supplementing experiment of mice by gavage

Control group (C group): normal mice, gavage 0.25 ml 3% PBS solution; diabetic group (D group): diabetic mice, gavage 0.25 ml 3% PBS solution; Metformin group (M group): diabetic mice, gavage 0.25 ml of 10 mg/kg.bw metformin; β -Hydroxybutyrate group (BHB group): diabetic mice, gavage 40mg/kg.bw [39]. The mice were given intragastric administration once daily from weeks 1 to 12.

Blood glucose testing

The blood was collected from the tail using a rapid blood glucose meter (Sannuo, China). Fasting blood glucose (FBG) was measured in mice overnight fasting for 12 h. Postprandial 2 h blood glucose (PBG) was measured in mice after eating for 2 h.

Oral glucose tolerance test (OGTT)

The mice were fasted overnight for 12 h. The initial blood glucose was measured (0 min), and then a 2 g/kg glucose solution was given to the stomach. The blood glucose values were measured at 30, 60, 90 and 120 min.

Samples collection

At the end of the experiment, the mice were fasted for 12 h and injected 100 mg/kg bw of ketamine into the intraperitoneal cavity after anesthesia. Blood was taken from the eyeballs, and then dislocated and sacrificed. The blood was centrifuged for 10 min at 3000×g at 4°C, and then the supernatant liquid was drawn and frozen at -80°C. The collected pancreatic tissues were fixed in 4% formaldehyde solution. The liver, intestinal tissue and intestinal contents were collect and freeze it at -80°C.

GLP-1 content detection

The colon contents were added the sample diluent and centrifuged for 10 min at 3000×g at 4°C. The supernatant liquid was drawn and the GLP-1 level was detected by ELISA kit (Nanjing Jiancheng, China), according to the instructions.

Determination of colonization of probiotic agents in intestinal tract of mice

L.plantarum YZX21 was incubated with 1 mL FITC fluorescent dye with a concentration of 1 mg/mL for 30 min at 37°C, washed with PBS until the liquid had no color, and diluted to 1×10^8 CFU/mL. The YZX21 group was gavaged the 0.5 mL of bacteria solution treated with dye. The mice were sacrificed at 1 h, 3 h, 6 h, and 12 h, and the entire digestive tract of the mice was taken and stored in liquid nitrogen. Intestinal tissue was photographed by IVIS Lumina XRMS Series III (PerkinElmer, Waltham, MA).

Widely-targeted metabolomics metabolic testing

The colon contents was thawed on ice. 50 mg of sample was taken and homogenized it with 500 ul of ice-cold methanol/water (70%, v/v). The sample was vortexed for 3 min, sonicated for 10 min in an ice water bath, and vortexed for 1 min. Then centrifuge it with 12,000×g at 4°C for 10 min.

The colon contents of the mice were analyzed using an LC-ESI-MS/MS system. The analytical conditions were as follows, UPLC: column, Waters ACQUITY UPLC HSS T3 C18 (1.8 μ m, 2.1 mm*100 mm); column temperature, 40°C; flow rate, 0.4 mL/min; injection volume, 5 μ L [40]. LIT and triple quadrupole (QQQ) scans were acquired on a triple quadrupole-linear ion trap mass spectrometer (QTRAP), QTRAP® LC-MS/MS System, equipped with an ESI Turbo Ion-Spray interface, operating in positive and negative ion mode and controlled by Analyst 1.6.3 software (Sciex). Metabolome measurements were carried out through a facility service at Metware Biotechnology Co., Ltd.

Determination of FFA in the colon

The contents of the mouse colon were taken, and a commercial kit (Solarbio, China) was used to detect the levels of FFA according to the instructions.

Histological analysis

HE staining: The mouse pancreas was fixed in tissue fixative for 24 h, and then stored in 70% ethanol solution. Part of the tissue was embedded in paraffin, and then stained with hematoxylin and eosin for histopathological analysis. The morphology was observed through a microscope (Olympus, Japan).

Immunofluorescence double labeling: The pancreas tissue was added insulin and glucagon primary antibody and incubated overnight at 4°C. DAPI was added to counter-stain the nucleus. The slides were mounted and sliced under a fluorescent microscope (NIKON ECLIPSE TI-SR, Japan) to observe and collect images.

Immunohistochemistry: The primary antibody GLP-1 was added to incubate overnight, and the secondary antibody was added to incubate at room temperature. The hematoxylin stained nuclei were thick, dehydrated, transparent, and mounted. The sections were observed under an upright microscope (NIKON ECLIPSE TI-SR, Japan) and the number of positive cells was recorded.

Detection of related genes in diabetic mice

Take the colon tissue of mice added Trizol (Invertrogen, USA) lysate to extract total RNA from the tissues. CDNA was synthesized using ReverTra Ace®qPCR RT Master Mix Reverse Transcription Kit (Invertrogen, USA). The primer forward and reverse sequences are shown in Table S2[41]. After adding SYBR Green (Invertrogen, USA), perform the reaction in a real-time PCR (Bio-Rad, USA). The relative levels of gene expression was detected by using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Data were presented as mean $\pm$ standard deviation. SPSS 21.0 and Prism 7.0 (GraphPad Software) were used for statistical analysis and figure drawing. One-way analysis of variance and Turkey's multiple comparison test were used to analyze the differences among the groups. $p < 0.05$ was considered statistically significant.

Abbreviations

Type 2 diabetes mellitus (T2DM), *Lactobacillus plantarum* L. plantarum, streptozotocin (STZ), Glucagon-like peptide-1 (GLP-1), β -Hydroxybutyrate (BHB), Free fatty acid (FFA), G protein-coupled receptor (GPR), Insulin intraperitoneal injection (ITT), Hydroxyl carboxylic acid receptor (HCA2), De Man, Rogosa and Sharpe (MRS), Fasting blood glucose (FBG), Postprandial 2 h blood glucose (PBG), Oral glucose tolerance test (OGTT)

Declarations

Ethics approval and consent to participate

The experiment was carried out in strict accordance with the “British Animal (Scientific Procedure) Act of 1986” (PPL 70/7652) and was approved by the Ethics Committee of Ocean University of China (SPXY2020060503).

Consent for publication

Not applicable.

Availability of data and materials

The data reported in this paper have been deposited in Mendeley Data: DOI: 10.17632/3hj8cbj32y.1

Competing interests

No conflict of interest exists in the submission of this manuscript, and manuscript is approved by all authors for publication.

Funding

This work was supported by Shandong Taishan industry leading talent project tscy2020xxxx and the National Key R & D of China (2018YFC0311201).

Authors' contributions

Lanwei Zhang designed and guided the experiments; Zhe Zhang wrote the original draft; Zhe Zhang, Xi Liang and Jingjun Tong performed the experiments; Youyou Lv, Haiyan Lu, and Maozhen Zhao analyzed the data; Pimin Gong, Tongjie Liu and Huaxi Yi revised the manuscript.

Acknowledgements

We acknowledge the laboratory of functional dairy and probiotics engineering and marine bioactive substances that have contributed to the research results reported in this work.

Authors' information

College of Food Science and Engineering, Ocean University of China, Qingdao 266003, China

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Figures

Figure 1

Effects of *L.plantarum* YZX21 on body weight, food intake, and blood glucose in T2DM mice. (A) The body weights were measured for 12-week (n=8 mice/group). (B) The food intake were measured for 12-week (n=8 mice/group). (C) Fasting glucose levels and (D) Postprandial blood glucose levels were

determined in each group (n=8 mice/group). (E) The OGTT and (F) AUCglucose was calculated for each group (n=8 mice/group). Data are shown as mean $\pm$ SD. Different letters indicate significance between columns ($p < 0.05$).

Figure 2

Effects of *L.plantarum* YZX21 on insulin secretion and pancreatic islets in T2DM mice. (A) ITT, (B) AUCglucose, (C) insulin level and (D) HOMA-IR calculated for each group (n=8 mice/group); (E)

Representative images of pancreas with HE stain were shown (n=6 mice/group). (F) Pancreatic islets were identified by insulin immunofluorescence (green), and glucagon was determined by TUNEL (red); nuclei were visualized by DAPI staining and shown in blue (n=6 mice/group). ICCD: islet cell cavitating degeneration; IPI: irregular pancreas islet; VP: vascular proliferation; MAPI: Medium autolysis pancreas islet; SAPI: slight autolysis pancreas islet. Data are shown as mean $\pm$ SD. Different letters indicate significance between columns ($p < 0.05$).

Figure 3

The colonize site of *L.plantarum* YZX21 and its effects on the intestinal GLP-1 secretion in T2DM mice. (A) Gastrointestinal fluorescent tracer of probiotic for 1 h, 3 h, 6 h, 12 h fluorescence imaging (n=3 mice/group).(B) Immunostaining showing GLP-1-positive cells in the colon (n=6 mice/group) (C) The number of GLP-1-positive cells in the colon (D) The levels of GLP-1 in colon (n=6 mice/group). (E) The mRNA expression of GCG in the colon (n=6 mice/group). (F) The mRNA expression of PC3 in the colon (n=6 mice/group). Data are shown as mean $\pm$ SD. Different letters indicate significance between columns (p < 0.05).

Figure 4

Effects of L.plantarum YZX21 on intestinal metabolites in mice. OPLS scores plot (A) and corresponding S-plot (B) of the D and L.plantarum YZX2l groups (n=8 mice/group). (C) BHB was the highest negative metabolites association with T2DM (n=8 mice/group). (E) and (F) Comparison of relative peak areas of BHB and FFA in the different groups (n=8 mice/group). Data are shown as mean $\pm$ SD. Different letters indicate significance between columns ($p < 0.05$).

Figure 5

Effects of BHB on blood glucose and pancreatic islets in T2DM mice. (A)FBG levels and (B)PBG levels were determined in each group (n=8 mice/group). (C)insulin levels and (D)HOME-IR were determined in each group (n=8 mice/group). (E) Representative images of pancreas with HE stain were shown (n=6 mice/group). (F) Pancreatic islets were identified by insulin immunofluorescence (green), and glucagon was determined by TUNEL (red); nuclei were visualized by DAPI staining and shown in blue (n=6 mice/group). ICCD: islet cell cavitating degeneration; IPI: irregular pancreas islet; VP: vascular proliferation; MAPI: Medium autolysis pancreas islet; SAPI: slight autolysis pancreas islet. Data are shown as mean $\pm$ SE. Different letters indicate significance between columns ($p < 0.05$).

Figure 6

Effect of BHB on GLP-1 expression. (A) Immunostaining for GLP-1 and (B) the number of GLP-1-positive cells in the colonic mucosa (n=6 mice/group). (C) The levels of GLP-1 in colon (n=6 mice/group). Data are shown as mean $\pm$ SE. Different letters indicate significance between columns ($p < 0.05$).

Figure 7

Effect of BHB on FFA and TG levels. (A) The effect of BHB on FFA level (n=6 mice/group), (B) The effect of BHB on TG level (n=6 mice/group). Data are shown as mean $\pm$ SE. Different letters indicate significance between columns ($p < 0.05$).

A

Figure 8

Effects of BHB on GPR109a-GPR41 and GPR43 expression. (A) Immunohistochemistry showing GPR109a in the colon (n=6 mice/group) (B)mRNA levels of GPR109a-GPR41 and GPR43 were determined in each group (n=6 mice/group). Data are shown as mean $\pm$ SD. Different letters indicate significance between columns ($p < 0.05$).

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