RESEARCH ARTICLE

Improvement of intestinal flora in a mouse model of diabetes mellitus by biotechnology: The case of *Lactobacillus* fermented chickpea milk

Shuming Chen*

Sanmenxia Key Laboratory of Biotechnology, Sanmenxia, Henan, China.

Received: December 14, 2023; **accepted:** March 4, 2024.

Diabetes mellitus' complications are serious and the common treatment drugs have many side effects. Therefore, research on new methods of lowering blood sugar is urgent. Chickpeas have a significant hypoglycemic effect and can be combined with lactic acid bacteria fermentation to improve quality. It is hoped to develop new functional foods. This study was to investigate the effect of *Lactobacillus* and chickpea on the control of diabetic mice based on their modulating effect on intestinal flora. 48 4-week-old male SPF mice were evenly divided into four groups including one normal control group (CN) and three diabetes experimental groups that were established by intraperitoneal injection of streptozotocin after 8 weeks of high-sugar and high-fat rearing. The experimental groups were continuously fed with high-sugar and high-fat food and were divided into non-intervention group (DM), unfermented chickpea milk group (CM), and fermented chickpea milk group (FCM). The activity, spirit, and appearance of the mice were recorded daily, and fasting blood glucose and body weight were measured weekly. At the end of the experiment, blood and organ samples were collected, and the intestinal flora in mouse feces was analyzed to assess the effect of chickpea milk on intestinal flora using Operational Taxonomic Units Clustering (OTUs) and species classification analysis. The results showed that CN group demonstrated excellent mental status, while DM group demonstrated reduced mental status. The mice were characterized by the typical symptoms of diabetes mellitus. Compared with the DM group, the intervention groups (CM and FCM) showed improved mental status and activity, closer to normal hair condition, less moist under pads, and reduced odor. Through the 6-week intervention period, the blood glucose level stabilized at around 7.0 mmol/L in CN group and above 23.0 mmol/L in the DM group. The abundances of *mucinophilic Eckermannia* in CN, DM, CM, and FCM groups were 9.7%, 6.1%, 9.0%, and 12.3%, respectively. The results showed that *Lactobacillus* fermented chickpea milk possessed effective intervention on the intestinal flora of diabetic mice, which provided guidance for effective intervention and care in the clinic.

Keywords: chickpea; diabetes mellitus; *Lactobacillus*; gut flora; biotechnology.

***Corresponding author:** Shuming Chen, Sanmenxia Key Laboratory of Biotechnology, Sanmenxia 472000, Henan, China. Emails: chenshumingg123@163.com.

Introduction

Diabetes mellitus is a chronic disease that is growing globally and is forecasted by the World Health Organization (WHO) to be the seventh main reason of death worldwide by 2030. Despite the availability of an extensive range of medications and treatment options, effective management of diabetes and its complications remains a major challenge. In recent years, there has been increasing evidence of a close relationship between gut flora and a variety of diseases, including diabetes. Ren *et al*. explored the pharmacological effects and mechanism of

action of 1-deoxynojirimycin (DNJ), a major alkaloid in mulberry (*Morus alba* L.), in pre-diabetic mice [1]. The results showed that DNJ significantly reduced blood glucose (BG) levels and enhanced insulin sensitivity in pre-diabetic mice, inhibited plasma levels of lipopolysaccharide (LPS), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) and altered intestinal bacterial composition. Matthieu *et al*. analyzed the intestinal flora (IFL) and mucosal health by means of 16S ribosomal RNA sequencing and other methods. The results found that, in a mouse model of type 1 diabetes (T1D), the intestinal mucosa had decreased levels of several key cytokines including IL-17A, IL-22, and IL-23A, impaired intestinal epithelial cell function, and was associated with an imbalance of the bacterial flora, in particular a decrease in the segmented filamentous bacterium SFB [2]. Zhao *et al*. found that oryza sativa polysaccharides (OsPs) had the ability to reduce type 2 diabetic (T2DM) mice's fasting blood glucose (FBG) levels, improved oral glucose tolerance test (OGTT), and decreased inflammatory factor (IF) levels, lipid accumulation in the liver, and insulin resistance in T2DM mice. In addition, OsPs adjusted the IFL imbalance in T2DM mice, especially increasing the abundance of *Lactobacillus* [3]. *Lactobacillus* and chickpea milk have attracted attention as potential diabetes management aids due to their low glycemic index (GI) and prebiotic properties. However, there is a lack of sufficient evidence on its long-term effects on glycemic regulation and safety in the clinical setting, while consumer acceptability of chickpea milk and individual variability of *lactobacilli* have been the focus of research. Therefore, further studies are needed to assess its utility and potential benefits as a functional food in diabetes treatment. Natsumi *et al*. tested the efficacy and safety of chickpea milk as a functional food in the treatment of diabetes mellitus using the lactic acid bacteria *Lactiplantibacillus pentosus* Himuka-SU5 (LpFCM) and *Lactococcus lactis* subsp. *lactis* Amami-SUl (LcFCM) fermented chickpea milk for antioxidant, anti-glycosylation, and bile acid lowering functions *in vitro*. The results showed that the anti-glycosylation in bovine serum albumin-fructose model as well as the bile acid lowering capacity of chickpea milk increased significantly after fermentation [4]. This research investigated the influence of *Lactobacillus* fermented chickpea milk on the IFL of diabetic mice and its hypoglycemic effect. The results of this study could provide the theoretical support for the further development of fermented chickpea products with diabetes adjuvant therapeutic function and improve the therapeutic care of diabetic patients.

Materials and methods

Preparation of fermented chickpea milk

The chickpeas were obtained from Yingge Biotechnology Co., Ltd. (Mulei, Xinjiang, China) and soaked overnight at room temperature, washed with distilled water, and churned with 10 times their weight of distilled water for 20 minutes at 100°C in a JR05-300 Cooking Machine (Zhejiang Supor Co., Hangzhou, Zhejiang, China) before autoclaved at 121°C for 20 minutes. The *Lactobacillus acidophilus* ATCC 4356 (Beijing Chuanglian Biotechnology Co., Ltd., Beijing, China) and *Lactobacillus plantarum* subsp. *plantarum* CICC 20279 (China Industrial Microbial Strain Preservation and Management Centre, Beijing, China) were activated in MRS liquid medium at 37℃ for 18 h continuously for 3 generations. The OD_{600} value was determined by using TV-1901 UV-vis spectrophotometer (Shanghai Precision Scientific Instruments Co., Shanghai, China). The viable bacteria were counted to determine the number of viable bacteria corresponding to the optical density. The re-activated bacterial solution was adjusted to the corresponding optical density, centrifuged at 4,000 rpm, 4℃, for 10 mins. The pellet was washed 2~3 times with saline, resuspended, and the suspension was uniformly adjusted to 1.0 x 10^8 colony forming unit (CFU)/mL according to the results of the viable bacterial counts. Four groups of chickpea milks were prepared as unfermentation, *L. acidophilus* fermentation, *L. plantarum* fermentation, and mixed

fermentation with 1% bacterial inoculum being added to corresponding sterilized and cooled chickpea milk and incubated in XMTD-204 digital display constant temperature oscillator (Jiangsu Jintan Yitong Electronics Co., Changzhou, Jiangsu, China) at 37°C, 160 rpm, for different fermentation hours.

Determination of unfermented and fermented chickpea milk indicators

(1) Total phenol content:

Both lyophilized unfermented and fermented chickpea milk powders were employed to determine their total phenol content based on the standard curve that was constructed by mixing 0, 0.4, 0.8, 1.2, 1.6, and 2.0 mL of 50 mg/L gallic acid standard solution with 1 mL of Folin-Phenol reagent for 10 min and then 3 mL of 7.5% sodium carbonate solution at 45°C for 1.5 h before measuring the absorbances at 765 nm.

(2) α-glucosidase inhibition:

Both unfermented and fermented chickpea milk lyophilized powders were mixed with p-nitrophenyl-β-D-glucopyranoside (PNPG), phosphate buffered saline (PBS), and 0.2 U/mL α-glucosidase to assess its inhibitory effect. The OD was measured at 405 nm. Four experiments were performed including (1) sample group: 50 mg lyophilized powder + 100 µL of α-glucosidase, (2) sample blank group: 50 mg lyophilized powder + 100 µL of PBS, (3) control group: 100 µL of α-glucosidase, (4) blank group: 100 µL of PBS.

(3) α-amylase inhibitory capacity:

Both unfermented and fermented lyophilized chickpea milk powders were mixed with soluble starch solution, PBS, and 5U/mL α-amylase. The OD was measured at 540 nm. Four experiments were conducted as (1) sample group: 50 mg lyophilized powder + 250 µL of α-amylase, (2) sample blank group: 50 mg lyophilized powder + 250 µL of PBS, (3) control group: 100 µL of α-amylase, and (4) blank group: 100 µL of PBS.

Construction of diabetes animal models

A total of 48, 4-weeks old, male, specific-pathogen-free (SPF), C57BL/6J mice (Animal Experimentation Centre, Zhengzhou, Henan, China) were included in this study. All animals were housed in SPF grade animal facility with 4 mice per cage at the room temperature of 18-24°C, humidity 55%, and 12 h alternating light during the feeding period to acclimatize for one week. The animals were then divided into 4 groups with 12 mice in each group including normal control group that was fed with normal chow, diabetic control (DM) group that was fed with high sugar and high fat (HSHF) chow, unfermented chickpea milk intervention group (CM) that was fed with HSHF and CM, fermented chickpea milk intervention group (FCM) that was fed with HSHF and FCM. Except for fasting before modeling, all groups had free access to food and drink. After 8 weeks of HSHF feeding, mice in DM, CM, and FCM groups received intraperitoneal injection of 50 mmol/L streptozotocin (STZ) prepared in sodium citrate buffer (pH 4.5) at the dosage of 100 mg/kg of body weight for three consecutive days. The normal control group received an equal amount of citrate buffer intraperitoneally. All mice were fasted for 12 hours on the third day after injection (water was allowed). On the following morning at 9: 00 am, blood was collected through the tail vein and FBG levels were determined. When the FBG values in HSHF feed groups exceeded 11.1 mmol/L for three consecutive days, the diabetes model was successfully established. However, If the model was not successfully established, supplemental injections of STZ would be performed. All the experimental procedures of this study were approved by the Medical Ethics and Laboratory Animal Welfare Committee (School of Medicine, Henan University, Zhengzhou, Henan, China).

Determination of the effects of chickpea milk

The corresponding experimental feeds were prepared according to the formulations in Table 1. The animals' activity, mental status, and appearance were recorded daily. Physiological indexes including FBG and body weight were measured every other week. All mice were fasted for 12 hours before measurement but were allowed to drink water. At the end of the sixth week of the intervention, mice were sacrificed by

Table 1. Experimental feeding formulation.

Materials	Normal feed (g/kg)	HSHF (g/kg)	CM (g/kg)	FCM (g/kg)
Unfermented Chickpea flour	0	0	100	0
Fermented Chickpea flour	0	0	0	100
Corn starch	397.5	234.5	173.5	173.5
Casein	200	118	95.3	95.3
Maltodextrin	132	77.88	77.88	77.88
Soya bean oil	70	41.3	41.3	41.3
Fructose	200	259	259	259
Fiber	50	29.5	29.5	29.5
Cysteine	3	1.77	1.77	1.77
Tert-Butylhydroquinone	0.014	0.083	0.083	0.083
Choline bitartrate	2.5	1.48	1.48	1.48
Multivitamin	10	5.9	5.9	5.9
Mineral complex	35	20.65	20.65	20.65
Barnyard	0	30	30	30
Lard	0	180	180	180

Notes: HSHF: high sugar and high fat feed. CM: unfermented chickpea milk feed. FCM: fermented chickpea milk feed.

decapitation after a 12-hour fast followed by blood sampling from the eyeballs. Blood samples were centrifuged at 3,500 rpm, 4°C, for 15 mins. The serum was collected and stored at -20°C. At the same time, kidney, liver, and spleen organs were removed and weighed, washed in saline, and dried. The organ index was then calculated by dividing the organ fresh weight with the mouse weight. Feces were also collected from the colon and stored at -80°C.

(1) Biochemical parameters and inflammatory factors:

Total cholesterol (TC) assay kit, triglyceride (TG) assay kit, high-density lipoprotein cholesterol (HDL-C) assay kit, and low-density lipoprotein cholesterol (LDL-C) assay kit were obtained from Nanjing Jiancheng Biological Engineering Research Institute of China (Nanjing, Jiangsu, China) to detect the levels of biochemical parameters in the serum of mice following the manufacturer's instructions. Serum levels of TNF-α, IL-6, and interleukin-10 (IL-10) were determined by using enzyme-linked immunosorbent assay (ELISA) kits (Nanjing Jianjian Bioengineering Institute, Nanjing, Jiangsu, China) following the manufacturer's instructions.

(2) Intestinal flora analysis:

Bacterial DNAs were extracted from mouse feces and tested for purity and concentration using the DNeasy PowerSoil Pro Kit (Qiagen, Venlo, Netherlands). 10 µL of DNA sample was diluted to 1 ng/uL. The 16s rRNA primers were designed using Oligo 7 software (OLIGO, Colorado Springs, CO, USA) and synthesized by Shanghai Sangong Biotechnology (Shanghai, China) (Table 2). The regular polymerase chain reaction was performed to amplify the bacterial 16s RNAs followed by DNA sequencing (Zhengzhou Huazhiyuan Medical Laboratory, Zhengzhou, Henan, China). Based on the sequencing results, the quantitative PCR (qPCR) was performed additionally to verify the accuracy of the high-throughput sequencing results for the top ten bacteria species at the family taxonomic level and relative abundance ratio. The qPCR reaction included 0.4 µL of each forward and reverse primers, 10 µL of 2× ChamQ Universal SYBR qPCR Master Mix (Shanghai Nuowei Zan Biotechnology Co., Shanghai, China), 1 µL of template DNA, and 8.2 µL of ddH_2O in a total volume of 20 µL. The qPCR was performed as 95°C for 30 s followed by 40 cycles of 95°C for 3 s and 60°C for 20 s, and then 65°C for 15 s with the dissolution curve from 60 -95°C using GeneAmp PCR System 9700 (Applied Biosystems, Carlsbad, California, USA).

Table 2. Primer sequences.

Primer name	Forward primer (5' – 3')	Reverse primer (5' – 3')
Lactobacillaceae	CGCATAACAACTTGGACCGAATGG	CTCAGGTCGGCTACGTATCATTGC
Bacteroidaceae	CGATGATACGCGAGGAACCTTACC	CGGCACGAGCTGACGACAAC
Muribaculaceae	GCTGCCTAAGCGGAACCTCTAAC	CCTTCGCCATCGGTGTTCTTCC
Xanthobacteraceae	CTAGCGTTGCTCGGAATCACTGG	CGCCTTCGCCACTGGTGTTC
Rikenellaceae	GATGCGGTAGGCGGAATGTATGG	TGGTAAGCTGCCTTCGCAATCG
Prevotellaceae	TGGTCAATGGACGCAAGTCTGAAC	CGGCTGCTGGCACGGAATTAG
Sphingomonadaceae	GCATCGCTTGAATCCAGGAGAGG	CCTTCGCCACTGGTGTTCTTCC
Bifidobacteriaceae	TCGAATAAGCACCGGCTAACTACG	GGCGCGGATCCACCGTTAAG
Paenibacillaceae	TCTTCCGCAATGGACGCAAGTC	CGGCTGCTGGCACGTAGTTAG
Lachnospiraceae	AGCTGGAGTGCAGGAGAGGTAAG	CGCCTTCGCCACTGGTGTTC
16S Universal	CCTACGGGAGGCAGCAG	ATTACCGCGGCTGCTGG

The raw data were spliced and filtered for operational taxonomic units (OTUs) clustering and species classification analysis. Based on the OTUs' results, the representative sequences of each OTU were annotated with species, and the top 10 species with the maximum abundance at each classification level were chosen for each group, while the relative abundance bar chart was produced. The significance of disparities in the species composition of the grouped samples was tested by using the Linear Discriminant Analysis Effect Size (LEfSe) method (https://huttenhower.sph.harvard.edu/lefse/).
The sequence data were analyzed and interpretated for microbial community composition by using Quantitative Insights into Microbial Ecology (QIIME) (http://qiime.org) and Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov).

Statistical analysis

SPSS 25 (IBM, Armonk, New York, USA) and Origin 2017 (OriginLab, Northampton, MA, USA) were employed in this study to analyze the data. All data were denoted as mean ± standard deviation. The difference between groups was determined by using Duncan's method or t-test with the *P* value less than 0.05 as the significant difference.

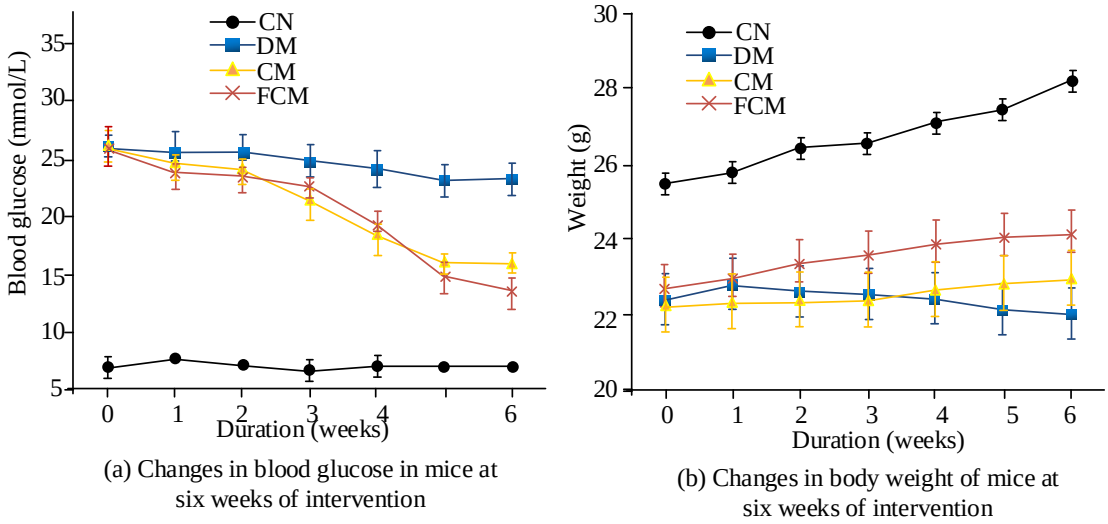
Results

The inhibition of α-glucosidase and α-amylase by fermented chickpea milk

Fermented chickpea milk showed higher α-glucosidase inhibition than that of unfermented chickpea milk, and chickpea milks fermented by *L. acidophilus* ATCC 4356 for 20 and 24 hours were particularly effective in inhibiting α-glucosidase with both inhibitions exceeding 80%. No significant difference was observed between the chickpea milks fermented for 20 and 24 hours ($P > 0.05$). However, there were significant differences between 20- and 24-hour fermentation groups and all the rest short time fermentation groups ($P < 0.05$). In contrast, although mixed fermented chickpea milk was also effective in inhibiting α-amylase, this inhibition was not as high as the inhibition of α-glucosidase and was below 40% in all fermentation groups. There was no significant difference between the blank control group and the *L. acidophilus* 20 h, 24h, and vegetative 20 h groups ($P > 0.05$), while the rest groups demonstrated significant differences ($P < 0.05$). Notably, the α-amylase inhibition of *L. plantarum* subsp. *plantarum* CICC 20279 fermented chickpea milk instead decreased. The results suggested that the inhibition ability of fermented chickpea milk to α-glucosidase was significantly better than that to α-amylase.

Comparison of animal growth and health status

The mice in each group demonstrated different growth and health status. Normal control group



(a) Changes in blood glucose in mice at six weeks of intervention

(b) Changes in body weight of mice at six weeks of intervention

Figure 1. The changes of blood glucose levels (BG) and animal body weights during the treatment period.

(CN) showed excellent mental status, high activity, and healthy shiny fur. The bottom pads were dry and had no odor. The mice were able to escape quickly from catching with a strong struggle. Mice in the non-intervention group (DM) showed reduced mental status, limited activity, dull and wet fur, wet underpads, and pungent odor. They were slow to escape and did not struggle significantly when being captured. The mice were characterized by the typical symptoms of diabetes mellitus with increased eating, drinking, urination, and less weight gain. Compared with the DM group, the intervention groups (CM and FCM) showed improved mental status and activity, hair condition closer to CN group, less moist underpads, and reduced odor. Especially in the FCM group, the performance of the animals was closer to that of the CN.

Comparison of blood glucose level and body weight at 6 weeks of intervention

Through the 6-week intervention period, the blood glucose level (BG) of the normal control group (CN) stabilized around 7.0 mmol/L, while the BG value of the diabetic model group (DM) stabilized above 23.0 mmol/L. The results demonstrated that the BG levels fluctuated in the CN and the DM groups. However, in the intervention groups of CM and FCM, the BG levels were continuously decreased from the 1st week of intervention and decreased even more after the 3rd week of intervention. The FCM group showed a more pronounced BG level decrease compared to the CM group after the 5th week intervention (Figure 1a). The changes of animal body weight in each group during the experiment period showed that the CN group demonstrated the highest basal body weight of 25.5 g and continuously increased the weight during the experiment period. On the other hand, the DM group showed a continuous decrease in body weight. Both intervention groups showed the trends of weight increase just as CN. However, by week 5, the FCM group gained more weight than the CM group (Figure 1b).

Comparison of organ indices

Changes in organ indices after experiment showed that liver and kidney indices of mice (LKIM) in DM group significantly exceeded those of CN group ($P < 0.05$), indicating that diabetic mouse had enlarged liver and kidney after modeling. Comparatively, the LKIM in the FCM group remained the same as those of the CN group without statistical difference ($P > 0.05$). There was no statistical difference in spleen indices among all the groups ($P > 0.05$) (Figure 2).

Comparison of lipid indexes

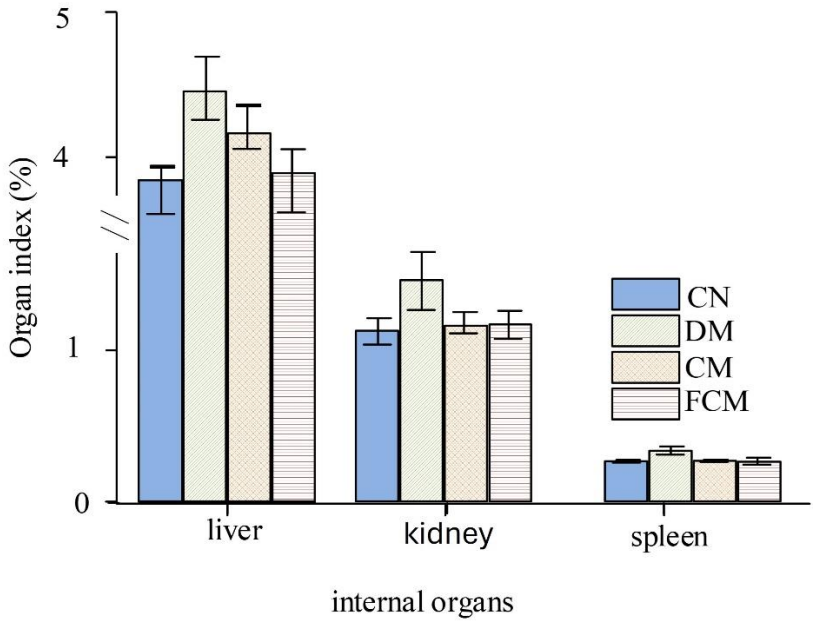


Figure 2. Animal organ indices of all experimental groups.

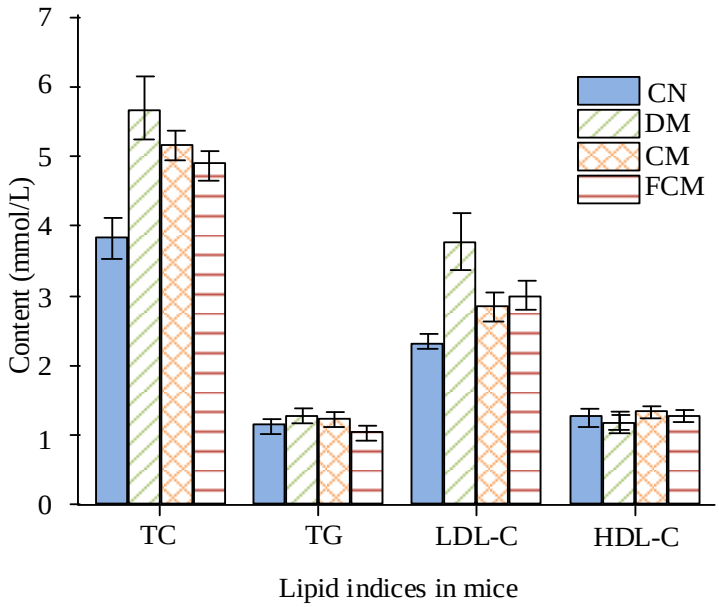


Figure 3. Comparison of lipid indices of all experimental groups.

The indices of triglyceride (TG) among all the groups showed no significant differences (Figure 3). However, the total cholesterol levels (TC) of the three diabetic groups (DM, CM, and FCM) were significantly exceeded the CN group ($P < 0.05$), with the TC level of the FCM group the closest one to the CN group. The LDL-C level of the DM group significantly exceeded the other three groups ($P < 0.05$), whereas there were no significant differences among the CM, FCM, and CN groups ($P > 0.05$). In addition, the HDL-C level in the CM group was significantly higher than that in the DM group ($P < 0.05$) (Figure 3).

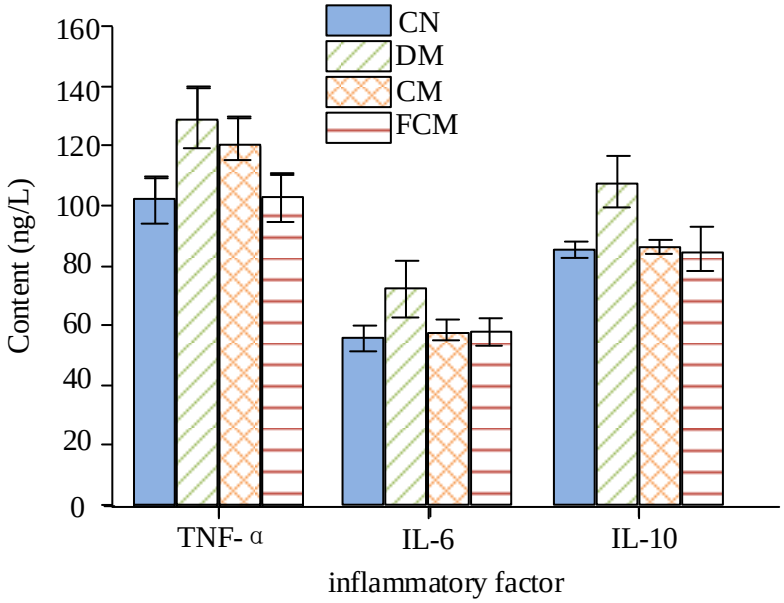
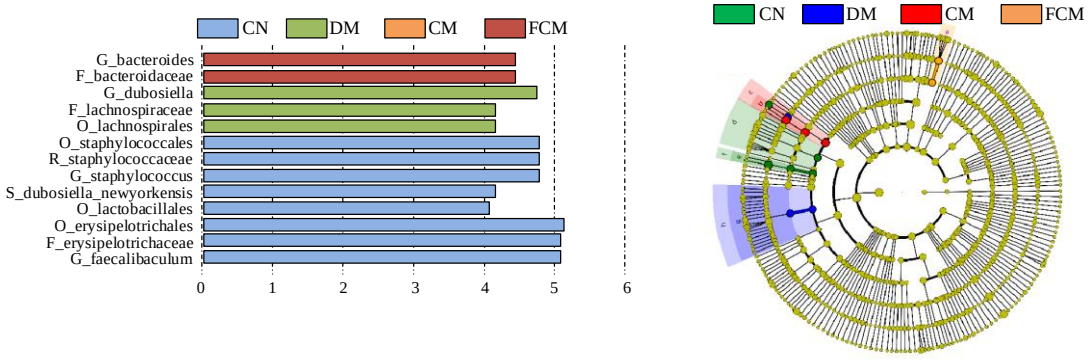


Figure 4. Comparison of serum inflammatory factors (Ifs) of all experimental groups.



(a) Histogram of the distribution of LDA values (b) Evolutionary branching diagrams

Figure 5. Analysis of intestinal flora (IFL) among experimental groups.

Comparison of serum inflammatory factors

The results showed that the serum levels of TNF-α, IL-6, and IL-10 in the DM group were significantly higher than that in the CN group ($P < 0.05$). Nevertheless, the levels of TNF-α, IL-6, and IL-10 in the FCM group showed no difference from the CN group (Figure 4).

Comparison of intestinal flora among groups

The characteristic microorganisms in the CN group were *Lactobacillales*, the probiotics maintaining intestinal health and discouraging colonization by pathogenic microorganisms. In the DM group, *Trichoderma sp*. and *Trichodermatidae* (*Lachnospirales* and *Lachnospiraceae*) were the main bacteria associated with hyperlipidemia and diabetes. The CM group showed that *Danitobacteria* and the family of *Danitobacteriaceae* (*Erysipelotrichales* and *Erysipelotrichaceae*) as well as the genus *Faecalibaculum* were the featured microorganisms, while *Dantoaceae* had unclear role and *Faecalibaculum* was beneficial for gut health. The FCM group demonstrated

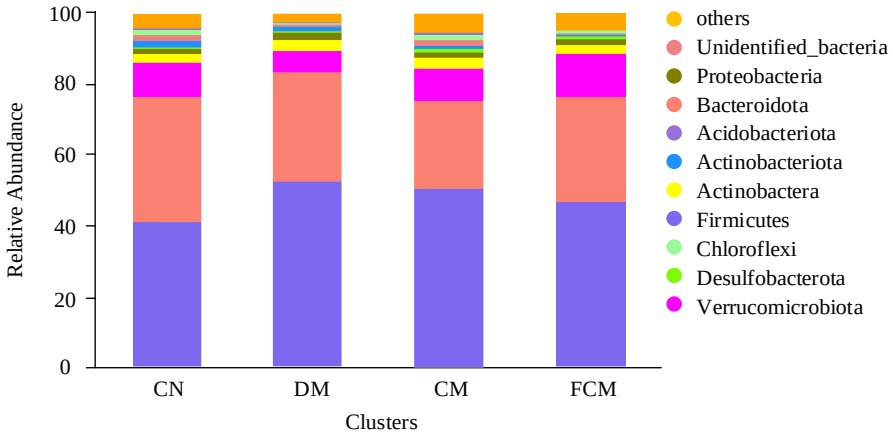


Figure 6. Comparison of intestinal flora among experimental groups.

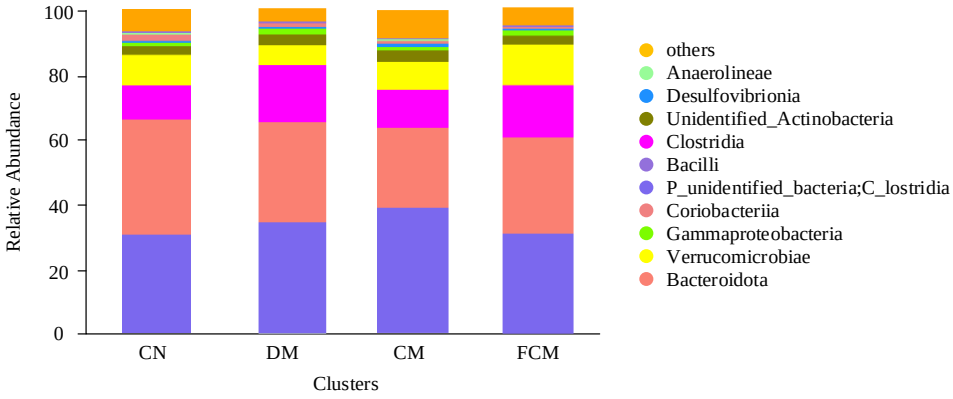


Figure 7. Different species composition of intestinal flora in each experimental group.

Bacteroidaceae and *Bacteroides* as characteristic bacteria. Both were second-generation probiotic bacteria with the ability to regulate T-cells and inhibit inflammation. The FCM group was also characterized by a combination of microorganisms from the family of *Bacteriophage* and *Bacteriophage* (Figure 5)

The species composition of intestinal flora in each group

The percentages of *Firmicutes*, *Bacteroidota*, and *Verrucomicrobiota* in the CN group were 41.0, 35.6, and 10.0%, respectively, while that in the DM group were 53.4, 30.8, and 6.1%, respectively. The percentages of the above three bacteria in the CM group were 50.6, 24.6, and 9.0%, respectively, while that in the FCM group were 46.8, 30.0, and 12.3%, respectively (Figure 6). The ratios of *Firmicutes*/Thick-walled *Bacteroidota* in the CN, DM, CM, and FCM groups were 0.87, 0.57, 0.48, and 0.64, respectively. The abundance of *Firmicutes*/*Proteobacteria* in the FCM group decreased to 1.75% compared to 1.93% in the DM group. Such a decrease helped to alleviate hyperglycemic symptoms.

The major components of the experimental animal's intestinal flora (IFL) at the phylum level including *Bacilli*, *Bacteroidia*, *Clostridia*, and *Verrucomicrobiae* were shown in Figure 7. In the DM group, the abundance of anaerobic *cordylobacteria phyla* was remarkably below the CN group ($P < 0.05$). However, in the FCM group, the abundance of anaerobic *cordyceps* showed no significant difference compared to that in the CN group, which suggested that fermented

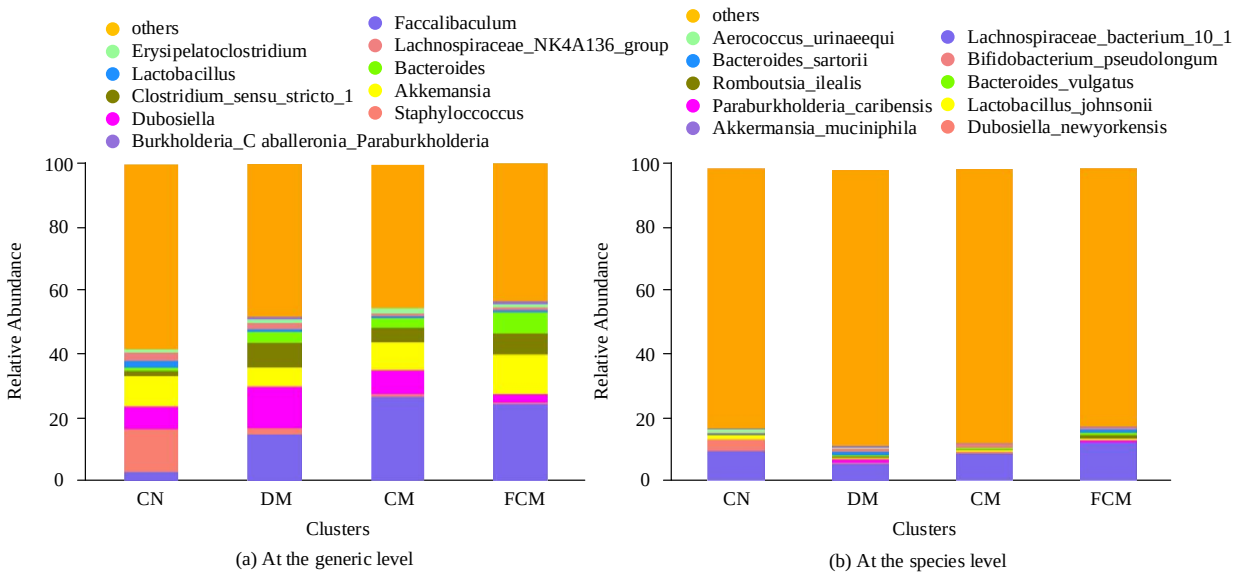


Figure 8. Comparison of IFL at the genus and species levels in all experimental groups.

chickpea milk could enhance the abundance of anaerobic cordyceps in the gut of diabetic mice, thereby contributing to slowing down weight loss in diabetic mice. At the genus level, the IFL of experimental groups demonstrated that *Faecalibaculum*, *Akkermansia*, *Staphylococcus*, and *Dubosiella* were the dominant microbial components. At the species level, *Akkermansia muciniphila* was the dominant species (Figure 8). The abundances of *Ekkermansia muciniphila* in the CN, DM, CM, and FCM groups were 9.7, 6.1, 9.0, and 12.3%, respectively.

Discussion

Gut flora is not only involved in nutrient absorption and digestion, but also influences the host's immune response and inflammatory state, which have been implicated in the pathogenesis of diabetes mellitus [5, 6]. Niu *et al*. evaluated the *in vivo* antidiabetic potentials of four *Cornus officinalis* (CF) extracts including saponin (CTS), orris glycoside (CIG), tannin (CT), and alcohol extracts (CCA). The results showed that all four extracts had therapeutic effects on diabetes by increasing body weight, lowering BG levels, and improving glucose tolerance. The CF extracts also promoted the production of short-chain fatty acids and modulated the composition of the intestinal microbiota [7]. *Lactobacilli* are a group of microorganisms widely used in food fermentation and preservation and have shown potential application in regulating intestinal microecological balance in recent years. Chickpea, as a nutrient-rich legume, is not only rich in protein and fiber, but also contains high levels of minerals and vitamins, as well as high levels of phenolics with hypoglycemic activity. Santos *et al*. studied the effect of chickpea flour and plant-based wheat gum (PSY) on the quality, sensory acceptability, glycemia, and satiety index of gluten-free bread (GFB) compared to the rice flour (RF) and tapioca starch (75:25) prepared control bread. The results showed that the combination of chickpea flour and PSY positively affected all the evaluated parameters, improved the nutritional content, and decreased the glycemic response of gluten-free breads with sensory appeal [8]. Sivapragasam *et al*. studied the fermentation process with blueberries, where the physical and chemical properties of blueberries were altered, contributing to the enhancement of organoleptic qualities, prolongation of shelf-life, and enhancement of health benefits. In addition, fermented

blueberries possess the potential for mitigating various non-communicable diseases including, but not limited to, diabetes, cancer, cardiovascular disease, cognitive disorders (neurodegenerative and neuropsychiatric disorders), and obesity compared to fresh blueberries [9]. This study analyzed the effect of *Lactobacillus* fermented chickpea milk on the intervention of diabetic mice. In the observation of the animal growth and status, after 6 weeks feeding, the animals in FCM group demonstrated better mental status and activity than that in the DM group. The mental status of FCM group was closer to that of the CN group. The BG of dietary intervention groups (CM and FCM) decreased significantly after the fourth week of intervention. The FCM group showed a better BG decrease than that in CM in the sixth week, but none of them reached the BG level of the CN. The body weights in the CM and FCM groups continued to increase like the CN group, and the FCM group increased more than that of the CM group by the fifth week. The results showed that both fermented and unfermented chickpea milk were effective in improving the symptoms of hyperglycemia and weight loss in type II diabetic mice, but the effect of fermented chickpea milk was more significant than unfermented chickpea milk.

In the comparison of organ indices, the LKIM in DM group significantly exceeded that in CN group ($P < 0.05$), while the LKIM in FCM group was similar to that in CN group without significant difference. There were no significant differences in spleen indices across all groups. The results indicated that FCM helped to improve the symptoms of enlarged liver and kidney in diabetic mice. However, due to the small size of spleen in the mouse, the short-term dietary interventions might not reflect the damage or repair of the spleen on the organ index. In a hyperglycemic environment, several body physiological indices demonstrated abnormalities including increased levels of BG, lipids, and proteins, as well as the appearance of glycosylation reactions. These changes have a significant impact on liver and kidney function [10, 11]. The liver's central role in glucose homeostasis is negatively affected by disturbances in glucose metabolism, as evidenced by inflammation and increased size. Similarly, the workload of the kidneys is increased under high glucose conditions, leading to glomerulosclerosis and increased volume. In the comparison of lipid indices in mice, the TC levels of mice in the DM, CM, and FCM groups significantly exceeded that in the CN group ($P < 0.05$), but the TC level of the FCM group was the closest one to the CN group. The LDL-C levels in the DM group also significantly exceeded that in the other three groups ($P < 0.05$). The HDL-C level in the CM group significantly exceeded that in the DM group ($P < 0.05$). The results indicated that FCM had a positive effect on lipid metabolism in diabetic mice. In the comparison of serum IFs, only the serum levels of TNF-α, IL-6, and IL-10 in the DM group significantly higher than that in the CN group ($P < 0.05$), which suggested that CM and FCM could effectively regulate the serum levels of IFs, thus inhibiting the progression of diabetes to a certain extent [12-14].

The FCM helped to improve the *Firmicutes*/ Thick-walled *Bacteroidota* (F/B) ratio from 0.57 (DM) to 0.64 (FCM) that was close to 0.87 in the CN group. The result implied that FCM had a positive impact in modulating the gut microbiome, especially in the ratio of proteobacteria to thick-walled bacteria [15]. It was evident that *Lactobacillus* fermented chickpea milk possessed good control and regulation of IFL in diabetic mice with the increase of the F/B ratio closing to the healthy control group, which might help to alleviate the symptoms of diabetes. The analysis of the abundance of anaerobic *cordyceps* in the intestinal tract showed that FCM enhanced the abundance of anaerobic cordyceps in the intestinal tract of diabetic mice, thereby contributing to slowing down weight loss in diabetic mice. The abundance of *mucinophilic Eckermannia* in different groups suggested that chickpea milk intervention could significantly increase the abundance of *mucinophilic Eckermannia* in the intestinal tract of diabetic mice and helped to alleviate the symptoms of

diabetes mellitus. Especially, the abundance in FCM group significantly higher than that in the other groups, which might indicate that FCM was one of the main factors for effective alleviation of diabetic symptoms [16, 17].

References

1. Ren X, Xing Y, He L, Xiu Z, Yang L, Han A, *et al*. 2022. Effect of 1-deoxynojirimycin on insulin resistance in prediabetic mice based on next-generation sequencing and intestinal. J Ethnopharmacol. 28(9):11-29.
2. Rouland M, Beaudoin L, Rouxel O, Bertrand L, Cagninacci L, Saffarian A, *et al*. 2022. Gut mucosa alterations and loss of segmented filamentous bacteria in type 1 diabetes are associated with inflammation rather than hyperglycaemia. Gut. 71(2):296-308.
3. Zhao Y, Song P, Yin S, Fan T, Li F, Ge X, *et al*. 2023. Onchidium struma polysaccharides exhibit hypoglycemic activity and modulate the gut microbiota in mice with type 2 diabetes mellitus. Food Funct. 14(4):1937-1951.
4. Handa N, Kuda T, Yamamoto M, Takahashi H, Kimura B. 2022. *In vitro* anti-oxidant, anti-glycation, and bile acid-lowering capacity of chickpea milk fermented with *Lactiplantibacilus pentosus* Himuka-SU5 and *Lactococcus lactis* subsp. *lactis* Amami-SU1. Process Biochem. 120(Sep):15-21.
5. Hao H, Li Z, Qiao S, Qi Y, Xu X, Si J, *et al*. 2023. Empagliflozin ameliorates atherosclerosis *via* regulating the intestinal flora. Atherosclerosis. 37(1):32-40.
6. Qu L, Liu C, Ke C, Zhan X, Li L, Xu H, *et al*. 2022. Atractylodes lancea *Rhizoma* attenuates DSS-induced colitis by regulating intestinal flora and metabolites. Am J Chinese Med. 50(2):525-552.
7. Niu D, An S, Chen X, Bi H, Zhang Q, Wang T, *et al*. 2020. *Corni Fructus* as a natural resource can treat type 2 diabetes by regulating gut microbiota. Am J Chinese Med. 48(6):1385-1407.
8. Santos FG, Aguiar EV, Rosell CM, Capriles VD. 2021. Potential of chickpea and psyllium in gluten-free breadmaking: Assessing bread's quality, sensory acceptability, and glycemic and satiety indexes. Food Hydrocolloids. 113(Apr):487-496.
9. Sivapragasam N, Neelakandan N, Rupasinghe HPV. 2023. Potential health benefits of fermented blueberry: A review of current scientific evidence. Trends Food Sci Technol. 13(2):103-120.
10. Hughes AE, De Franco E, Globa E, Zelinska N, Hilgard D, Sifianou P, *et al*. 2021. Identification of GCK-maturity-onset diabetes of the young in cases of neonatal hyperglycemia: a case series and review of clinical features. Pediatric Diabetes. 22(6):876-881.
11. Udler MS, Powe CE, Austin-Tse CA. 2020. Case 6-2020: A 34-year-old woman with hyperglycemia. Medicine. 382(8):745-753.
12. Li H, Zhang H, Zhao H. 2023. Apigenin attenuates inflammatory response in allergic rhinitis mice by inhibiting the TLR4/MyD88/NF-kappa B signaling pathway. Environ Toxicol. 38(1/2):253-265.
13. Temu TM, Zifodya JS, Polyak SJ, Wagoner J, Wanjalla CN, Masyuko S, *et al*. 2021. Antiretroviral therapy reduces but does not normalize immune and vascular inflammatory markers in adults with chronic HIV infection in Kenya. AIDS. 35(1):45-51.
14. Jiang W, Chen J, Gong L, Liu F, Zhao H, Yan Z, *et al*. 2023. Microbiota-derived short-chain fatty acids may participate in post stroke depression by regulating host's lipid metabolism. J Psychiatr Res. 161:426-434.
15. Takezawa K, Fujita K, Matsushita M, Motooka D, Hatano K, Banno E, *et al*. 2021. The *Firmicutes*/*Bacteroidetes* ratio of the human gut microbiota is associated with prostate enlargement. Prostate. 81(16):1287-1293.
16. Pedersen FN, Stidsen JV, Rasmussen MN, Nielsen HB, Henriksen JE, Olesen TB, *et al*. 2023. Retinal microvascular markers in type 2 diabetes subphenotypes and latent autoimmune diabetes of adults. Acta Ophthalmol. 101(5):560-567.
17. Russell WE, Bundy BN, Anderson MS, Cooney LA, Gitelman SE, Goland RS, *et al*. 2023. Abatacept for delay of type 1 diabetes progression in stage 1 relatives at risk: A randomized, double-masked, controlled trial. Diabetes Care. 46(5):1005-1013.