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## Flaxseed powder supplementation in non-alcoholic fatty liver disease: a randomized controlled clinical trial

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**Background:** Non-alcoholic fatty liver disease (NAFLD) has become a growing public health problem worldwide, and dietary interventions have important potential in the prevention and treatment of NAFLD. Moreover, previous animal studies have shown that flaxseed has a good improvement effect in animal NAFLD models. **Objectives:** Assess whether flaxseed powder could improve the liver lipid content in patients with NAFLD. **Methods:** In this 12-week randomized controlled clinical trial, 50 patients were randomly assigned to the flaxseed group ( $n = 25$ ) and the control group ( $n = 25$ ). The flaxseed group received 30 g d<sup>-1</sup> flaxseed powder orally before lunch or dinner along with health education, while the control group received only health education. The primary outcome was the intrahepatic lipid content assessed by the proton density fat fraction estimated by magnetic resonance imaging, and secondary outcomes were body composition measurements, liver function, and glucolipid metabolism. **Results:** Patients in the flaxseed group showed significantly lower liver fat content, body fat percentage, obesity index, visceral fat area, serum total bilirubin (TBIL), direct bilirubin (DBIL), indirect bilirubin (IBIL), aspartate aminotransferase (AST), total cholesterol (TC), and triglyceride (TG) levels after a 12-week intervention compared to pre-intervention levels, while serum apolipoprotein A1 (Apo A1) and high-density lipoprotein cholesterol (HDL-C) levels were significantly increased, with all differences being statistically significant ( $P < 0.05$ ). Analysis of the gut microbiota showed that, at the phylum level, flaxseed intervention significantly increased the abundance of *Bacteroides* and *Actinobacteria*, while decreasing the ratio of *Firmicutes* to *Bacteroidetes*. At the genus level, the relative abundance of *Clostridium\_sensu\_stricto\_1*, *Parasutterella*, *Lachnospiraceae\_NK4A136\_group*, *Eubacterium\_xylanophilum\_group*, and *Bifidobacterium* in the gut microbiota of the flaxseed group was significantly higher than that of the control group ( $P < 0.05$ ), whereas the relative abundance of *Coriobacteriaceae\_UCG-002* was significantly lower than that of the control group ( $P < 0.05$ ). **Conclusions:** Flaxseed powder intervention for 12 weeks had the effect of improving liver lipid deposition, liver function, body composition indicators, and lipid metabolism in patients with NAFLD. It also regulated the gut microbiota in NAFLD patients, increasing the abundance of beneficial bacteria while reducing harmful bacteria. This suggested that flaxseed is one of the natural and effective foods for improving NAFLD.

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## Introduction

Non-alcoholic fatty liver disease (NAFLD) is one of the most common chronic liver diseases globally, affecting 38% of the population, characterized by the accumulation of fat in the

liver.<sup>1</sup> The pathologic stages of NAFLD include nonalcoholic fatty liver (NAFL), nonalcoholic steatohepatitis (NASH), followed by the development of liver fibrosis and cirrhosis, and finally leading to the development of hepatocellular carcinoma.<sup>2</sup> Among them, about 1/5 of NAFLD patients show stage development into NASH, which is a major cause of cirrhosis and liver cancer.<sup>3</sup> With the rapid development of the social economy over the past few decades, the prevalence of NAFLD continues to rise. A meta-analysis involving 8.5 million people showed that the global prevalence of NAFLD increased from 25.3% in 2000 to 38.0% in 2019.<sup>4</sup> Furthermore, the increasing prevalence of obesity among young people exposes the popu-

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lation to NAFLD at an earlier age, and the prolonged course of the disease leads more individuals to develop liver fibrosis and cirrhosis.<sup>5</sup> Although progress has been made in research on the treatment of NAFLD, there is currently no effective clinical drug regimen for NAFLD. Recent studies have found that dietary structure is closely related to the occurrence of NAFLD. Poor dietary habits are an important cause of NAFLD, and regulation of dietary structure, such as energy restriction and rational nutrition, could reduce metabolic syndrome.<sup>6</sup> The Mediterranean diet, low-carbohydrate diet, and diets rich in fruits, nuts, and whole grains had a significant positive impact on improving NAFLD, providing valuable guidance and insights for the prevention and treatment of related diseases.<sup>7–12</sup>

Flaxseed, as a nutritionally rich food, has a long history of cultivation in Europe and Asia. Due to its high content of ALA, lignans, and dietary fiber, it has become an important functional food ingredient.<sup>13</sup> ALA not only helps improve blood lipids and blood pressure, but it also inhibits inflammatory reactions, relieves platelet aggregation, and helps prevent thrombus formation.<sup>14–16</sup> n-3 PUFA could reduce intrahepatic lipid accumulation, improve liver enzyme levels, increase insulin sensitivity, and exhibit anti-inflammatory effects.<sup>17,18</sup> Lignans are one of the most important and abundant phytochemicals in flaxseed, proven to offer a range of health benefits, including anti-cancer, antioxidant, neuroprotective, cardioprotective, and estrogenic properties.<sup>19–21</sup> Flaxseeds contain more than 10% soluble dietary fiber; consuming sufficient dietary fiber could reduce plasma triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and plasma cholesterol in rats, alleviating atherosclerosis.<sup>22</sup> In addition, dietary fiber supplementation could improve the gut microbiota in NAFLD patients, producing short-chain fatty acids to slow the progression of NAFLD.<sup>23,24</sup> Given that flaxseeds are rich in bioactive substances, the American National Cancer Institute has listed them as one of the six major plants targeted for cancer research.<sup>25,26</sup> However, the current nutritional research on flaxseed is mainly based on a single component of flaxseed, but the preparation and extraction of a single component of the process is complex and expensive and has small functional coverage. Whole flaxseed, as an organic whole, contains a wider variety of active ingredients, offers more comprehensive coverage, is easy to consume, and has a higher value for application and promotion.

In this article, we designed a randomized controlled clinical trial to evaluate the effectiveness of supplemental flaxseed powder as an adjunct intervention to a healthy diet in the treatment of NAFLD. The primary outcome was the liver fat content assessed by magnetic resonance imaging that estimated the proton density fat fraction (MRI-PDFF), while secondary outcomes included liver function, glucose–lipid metabolism, anthropometric measurements, and gut microbiota. This study aimed to explore the impact of flaxseed powder on mild to moderate NAFLD, providing new theoretical insights into the relationship between flaxseed and human health.

## Methods

### Participants and study design

This was a 12-week randomized controlled clinical trial (open-label), conducted from September 2022 to September 2023 at the People's Hospital of Lianshui County, Huai'an City, Jiangsu Province, China. The flaxseed was golden flaxseed purchased from Canmar Foods Ltd in Canada and contained the following per 100 g: protein – 20.0 g, fat – 48.7 g (saturated fatty acid – 3.3 g, polyunsaturated fatty acid – 40.0 g [omega-3 polyunsaturated fatty acid – 33.3 g and omega-6 polyunsaturated fatty acid – 6.7 g], monounsaturated fatty acid – 5.3 g and trans fatty acid – 0 g), carbohydrate – 20.0 g (dietary fiber – 20.0 g and sugar – 0 g), cholesterol – 0 g, sodium – 66.7 mg and lignan – 1.6 g. This trial was not blinded because it was difficult to find a placebo with the same color, smell, and characteristics. The study protocol was approved by the Ethics Approval Committee of Zhongda Hospital Affiliated to Southeast University (approval no. 2022ZDSYLL321-P01), and the study received informed consent and signed informed consent forms from all participants. The trial was registered at <https://www.chictr.org.cn> as ChiCTR2400091299.

From October 1st to 30th, 2022, volunteers were recruited at the Outpatient and Physical Examination Center of Lianshui County People's Hospital. Based on previous studies,<sup>27–29</sup> the largest sample size was calculated using the MRI-PDFF value as the dependent variable, with an expected difference in MRI-PDFF of 30%, assuming a two-sided  $\alpha$  level of 0.05, a power of 80%, and a sample size of 25 patients in each group, taking into account a 10% dropout. Local residents aged between 18 and 65 diagnosed with NAFLD through previous MRI-PDFF examination (MRI-PDFF  $\geq$  5%)<sup>30,31</sup> and willing to participate in the study were eligible. Exclusion criteria included: (1) patients with severe NAFLD (MRI-PDFF > 25%); (2) patients with diabetes, cardiovascular disease, digestive disorders affecting absorption, organ transplants, organ failure, malignant tumors and genetic diseases, and those using immunosuppressants; (3) those who had previously or currently suffered from viral hepatitis, drug-induced liver disease, autoimmune liver disease, and other conditions that could lead to fatty liver; (4) weight loss of >5% of their body weight in the past 6 months or currently following a weight loss diet; excessive alcohol consumption (women consuming >70 g of ethanol per week, men consuming >140 g of ethanol per week); (5) women during pregnancy, lactation, and menopause; and (6) history of flaxseed allergy, intake of nutritional supplements, probiotics, prebiotics, antibiotics, and proton pump inhibitors within 3 months, and consumption of large amounts of nuts, flaxseed, and sesame.

After completing the baseline assessment, 54 eligible NAFLD patients officially participated in the trial. Based on the patients' age, gender, body mass index (BMI), nutrient intake, liver fat content, and other indicators, 54 eligible subjects were randomly divided into 2 groups, flaxseed group ( $n = 27$ ) and control group ( $n = 27$ ), by a statistical analyst who was not involved in this study using SPSS 22.0. Neither the subjects



nor the researchers were aware of the random allocation sequence until the grouping was completed. The flaxseed group received 30 g d<sup>-1</sup> flaxseed powder orally before lunch or dinner along with health education, while the control group received only health education. The content of health education included distributing oil-limiting bottles and NAFLD health education manuals to subjects, as well as providing weekly health knowledge related to NAFLD. Subjects were surveyed in weeks 0 and 12 with questionnaires (sociodemographic information, general health information, and 3-day 24 h food recall), physical examinations (height, weight, waist circumference, hip circumference, and body composition measurements), laboratory tests, and MRI-PDFF, while also collecting fecal samples to assess changes in the composition of the gut microbiota. In this study, the 3-day 24 h food recall questionnaire was collected by professional nutritionists through face-to-face interviews. The nutritionists provided three dietary record tables, and participants randomly selected two weekdays and one weekend to record all food and beverages consumed. During the questionnaire filling, the nutritionists provided daily food models to assist participants in better understanding the survey and estimating food weights more accurately. After completing the food records, the nutritionists checked the accuracy and completeness of all the recorded information. The 3-day 24 h dietary survey results were then input into the nutrition calculator developed by the Nutrition and Health Institute of the Chinese Center for Disease Control and Prevention (CDC) to calculate the participants' daily dietary intake and analyze the nutrient content of the diet. Throughout the entire trial period, a revisit and retrieval of packaging bags was conducted every 10 days to observe any adverse reactions in the subjects and assess adherence, to ensure the quality of the trial, and flaxseed powder was distributed every 30 days.

#### Determination of the intrahepatic fat content by MRI-PDFF

The main outcome of this study was determined by measuring the change in the percentage of hepatic steatosis using MRI-PDFF in the participants. All examinations were conducted at the Imaging Department of the Lianshui County People's Hospital using the General Electric (GE) company's SIGNA Architect 3.0 T MRI scanner. The same MRI physician performed all MRI-PDFF examinations on the participants, utilizing an abdominal coil in combination with respiratory gating and segmental breath-holding to execute the MRI scans. Subjects should fast for 4 hours before liver MRI to minimize motion artifacts caused by excessive gastrointestinal peristalsis during the examination. Additionally, any metal objects should be removed from the body before the examination to ensure uniformity of the magnetic field. During the examination, the subject should lie supine, and the subject should hold their breath to cooperate with the examination. Three areas with fewer blood vessels, uniform liver texture, and away from the liver edge should be selected for measurement during the examination to increase the accuracy of fat quantification. Each scan should start at the end of inhalation

to minimize the interference of respiratory movements. After the scan was completed, the liver fat content of three areas of the liver texture was obtained, and the maximum value was taken to calculate the overall liver fat content, quantitatively calculating the grade of fatty liver.

#### Basic information collection and anthropometric assessment

At the beginning of the trial, the researchers collected the subjects' socio-demographic and general health information through face-to-face interviews, including age, gender, occupation, education level, smoking history, alcohol consumption history, daily activity level, history of previous diseases, previous medication use and so on. In addition, 3-day, 24-hour dietary recall data and dietary frequency questionnaires were collected consecutively by nutrition professionals at the beginning and end of the trial, supplemented by daily food modeling to help subjects estimate food weights more accurately. The results of the dietary survey were then entered into the FeiHua Nutrition Calculator software to calculate the daily dietary intake of the subjects and to analyze the nutrient intake. The DBA-450 Body Composition Analyzer was used for body composition measurement based on the bioelectrical impedance measurement method, and a Meilen MSG005-H ultrasonic measuring instrument was used to measure the height, with participants removing shoes, socks, outerwear, hats, and other heavy clothing before measurement to ensure the accuracy of the measurement results. A uniform standard soft ruler was provided to measure the waist circumference and hip circumference, and the average of the two measurements was taken as the final value.

#### Serum sample collection and measurement

Peripheral venous blood samples from the subjects were collected for biochemical testing before and at the end of the experiment. Subjects maintained a light diet the day before blood collection and fasted on food and water for 8 hours before blood collection. Blood samples were collected by medical personnel between 8:00–9:00 a.m. at the Health Examination Center of Lianshui County People's Hospital, and then sent to the Laboratory Department of Lianshui County People's Hospital for the measurement of blood routine, blood glucose and lipid metabolism related indexes, and liver function indexes within half an hour after the blood collection. Biochemical parameters related to glucose, liver function, renal function, inflammation, and lipid profile were measured using a Cobas 8000 automated biochemical analyzer, while fasting insulin (FINS) levels were measured using a MAGLUMI 400 PLUS fully automated chemiluminescence meter. All biochemical assessments were performed in the same laboratory using standard methods.

#### Fecal sample collection and microbial DNA extraction and sequencing

At the baseline and week 12, fecal samples from subjects were collected for sequencing. The fecal collection process followed the International Human Microbiome Standard (IHMS) procedure for fecal collection, and the collected fecal samples were stored in a refrigerator at –80 °C until testing. A commer-



cial DNA extraction kit (NobleRyder) was used to extract bacterial DNA from microbial samples obtained from the subjects, utilizing cetyltrimethylammonium bromide for the process. Primers 341F (5'-CCTACGGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') were used to amplify the 16S rRNA gene, especially the V3–V4 region. The PCR amplification conditions were as follows: initial denaturation at 98 °C for 30 s, then denaturation at 98 °C for 10 s, annealing at 54 °C for 30 s, and extension at 72 °C for 45 s in 32 cycles. The PCR amplification conditions were as follows: initial denaturation at 98 °C for 30 s, denaturation at 98 °C for 10 s, annealing at 54 °C for 30 s, extension at 72 °C for 45 s, a total of 32 cycles, and finally extension at 72 °C for 10 min. Ultrapure water was used throughout as a negative control to exclude false positive experimental results. Then the PCR products were purified using 2% agarose gel electrophoresis. The size and number of the amplified library were assessed using an Agilent 2100 Bioanalyzer (Agilent, USA) and an Illumina library quantification kit (Kapa Biosciences, Woburn, MA, USA). The sequencing platform used was the NovaSeq PE250 platform, with the selected instrument being the NovaSeq 6000 sequencer, and the matching reagent was the NovaSeq 6000 SP Reagent Kit (500 cycles).

### Statistical analysis

The questionnaires were entered using the EPIDATA version 3.1 software with double entry, and the data were processed and analyzed using SPSS 22.0 (version 22.0, SPSS Inc., Chicago, IL, USA). Quantitative data were analyzed based on the Shapiro–Wilk test. Normally distributed data were described as means  $\pm$  SD, and the Student's *t*-test was used to analyze differences between the two groups. For non-normally distributed data, it was described as the median (25th percentile, 75th percentile), and the Mann–Whitney *U* test was used to analyze and compare the differences between the two groups of data. Qualitative data were analyzed using the chi-square test and described in terms of composition ratios. In statistical analysis, the significance level was set at 0.05, and if  $P < 0.05$ , the difference between the two groups was considered statistically significant.

The software used for gut microbial diversity analysis was Qiime 2, which could calculate both microbial alpha and beta diversity, and inter-group variability was analyzed using the Student's *t*-test or the Wilcoxon test. Inter-group difference analysis was conducted using either the Student's *t*-test or the Wilcoxon test, and the Spearman rank test was used to compute the correlation between changes in participants' clinical outcomes and relative abundance changes in the gut microbiota. R3.5.2 (R Core Team) and GraphPad Prism 9.0 were used for chart plotting.

## Results

### Patient flow, tolerability, and compliance

The flowchart of the trial is shown in Fig. 1. At the beginning of the trial, 102 patients with NAFLD registered to participate.

Among them, 32 patients did not meet the inclusion criteria, 4 could not be contacted, 6 refused to attend medical examinations on time, and 3 were unable to control their diet and could not take the flaxseed on time. After screening, 54 individuals enrolled in the randomization, with 27 in each of the flaxseed group and the control group. In the flaxseed group, 2 participants discontinued the study, one was lost to follow-up and one was hospitalized due to a fracture. In the control group, 2 participants also discontinued the study, 1 was transferred for work reasons to another location, and 1 was diagnosed with breast cancer requiring surgery. No adverse reactions were reported during the trial, and the participants showed good compliance. Among those who completed the final follow-up, the compliance rate for daily flaxseed intake exceeded 90%.

### Baseline characteristics

The baseline sociodemographic, anthropometric, daily dietary intake, physical activity level, liver fat content, liver function, lipid profile, blood glucose, and FINS data of the subjects are shown in Table 1. There were no significant differences between the flaxseed group and the control group on all indicators at the baseline.

### Dietary energy and nutrient intake, and physical activity levels of the subjects

Table 2 shows the nutritional intake and physical activity of the flaxseed group and the control group at the baseline and week 12; there were no significant differences between the two groups in terms of energy, nutrition, or physical activity.

### Effects of flaxseed powder consumption on body composition

As shown in Table 3, after 12 weeks of flaxseed intervention, there was a significant reduction in body fat percent ( $P = 0.001$ ), obesity degree ( $P = 0.008$ ), and visceral fat area ( $P = 0.017$ ) compared to the baseline. Based on the analysis results of the differences between the baseline and 12 weeks, the subjects in the flaxseed group showed a mean decrease weight loss of 2.4 kg and a 6.4% reduction in body fat percentage, in addition to a significant decrease in the visceral fat area. However, there were no differences observed between the two groups in terms of skeletal muscle mass.

### Effect of flaxseed powder consumption on biochemical indicators

As shown in Table 4, both the liver function indicators of the flaxseed group and the control group subjects improved, but the improvement was more pronounced in the flaxseed group. In terms of liver function indexes, serum total bilirubin (TBIL) and direct bilirubin (DBIL) in both the flaxseed group and control group decreased significantly at 12 weeks with statistical differences, while indirect bilirubin (IBIL) and aspartate aminotransferase (AST) decreased significantly only in the flaxseed group, and the control group also showed a decreasing trend, but with smaller decreases. No significant changes in alanine aminotransferase (ALT) and cholinesterase (CHE) were observed before and after the intervention in both groups. In



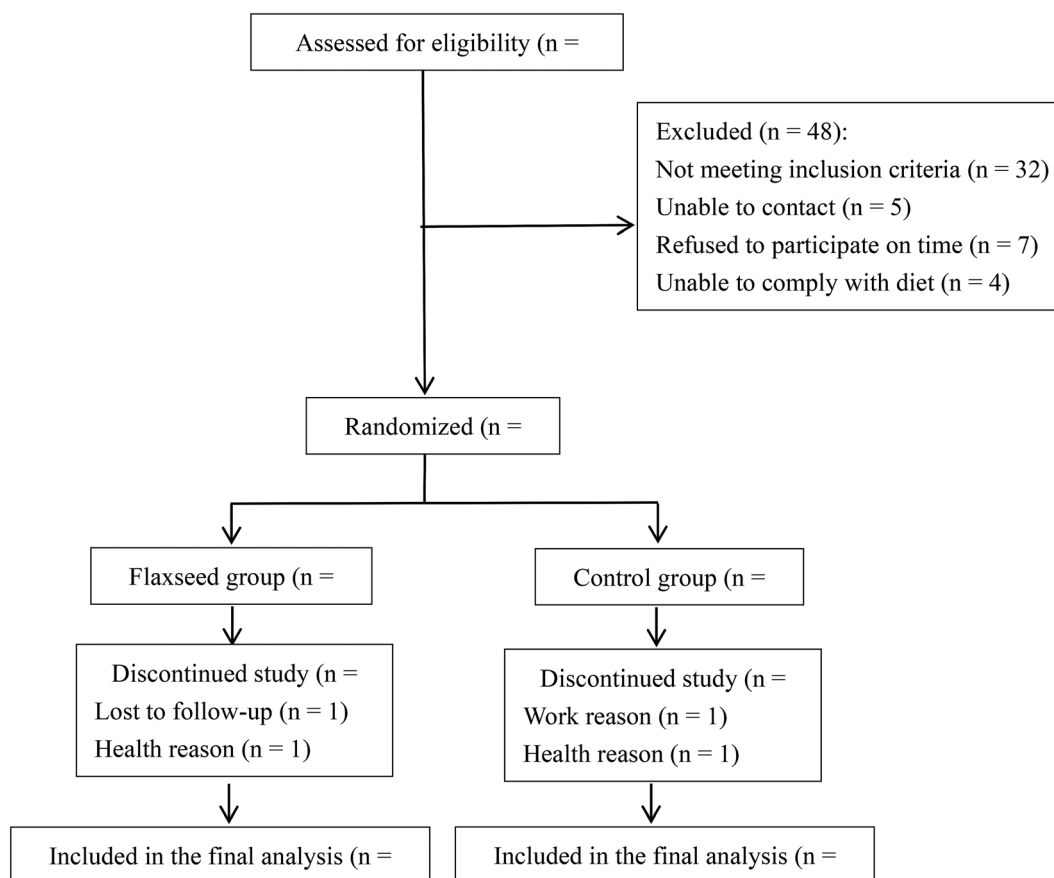


Fig. 1 Participant flowchart of the study.

terms of lipid profiles, there were also statistically significant differences ( $P < 0.05$ ) between the two groups in TG, HDL-C, and ApoA1 after 12 weeks of intervention. Although there was no statistical difference in the baseline and 12-week LDL-C levels between the two groups, the flaxseed group showed a reduction of  $0.23 \text{ mmol L}^{-1}$  from the baseline, while the control group only showed a decrease of  $0.05 \text{ mmol L}^{-1}$ . After 12 weeks of intervention with flaxseed powder, the total cholesterol (TC), TG, high-density lipoprotein cholesterol (HDL-C), and apolipoprotein A1 (Apo A1) levels of subjects in the flaxseed group improved significantly compared with the baseline. In addition, the flaxseed group showed a decrease in TG by  $0.4 \text{ mmol L}^{-1}$  from the baseline ( $P < 0.05$ ), and an increase in HDL-C and ApoA1 by  $0.10 \text{ mmol L}^{-1}$  and  $0.12 \text{ mmol L}^{-1}$  from the baseline ( $P < 0.05$ ), respectively, while the control group showed minimal changes compared to the baseline. However, no beneficial effects of flaxseed powder intervention were observed in glycemic control parameters.

#### Effect of flaxseed powder consumption on liver fat content

After 12 weeks of intervention, the comparison of differences in MRI-PDFF between the two groups also showed a statistically significant difference ( $P = 0.001$ ) (Table 5). Furthermore, the liver fat content of the participants in the flaxseed group

decreased by an average of 5.28% compared to the baseline ( $P = 0.006$ ). Although the liver fat content in the control group also decreased by 1.23%, there was no statistically significant difference ( $P = 0.225$ ) (Table 5). Further subgroup analysis results (Table 5) showed that flaxseed powder intervention did not find significant improvement in MRI-PDFF in 5%–10% of NAFLD subjects, although there was a downward trend, but no statistical difference. In contrast, in NAFLD participants with MRI-PDFF levels between 10% and 25%, flaxseed powder showed a positive intervention effect on the liver fat content; the liver fat content of the participants in the flaxseed group decreased by an average of 8.87% from the baseline ( $P = 0.006$ ). The liver fat content of each subject during the trial is shown in Fig. 2. Fig. 3 shows MRI-PDFF images of the liver fat content in 2 subjects. Fig. 3A and B show comparisons of the liver fat content at the baseline and at 12 weeks in 1 randomly selected subject in the flaxseed group. The liver fat content of the participant in the flaxseed group (male, 37 years old) decreased from 23.10% at the baseline to 15.17% at 12 weeks. Fig. 3C and D show comparisons of the liver fat content at the baseline and 12 weeks for 1 randomly selected subject in the control group, which increased from 17.00% at the baseline to 18.20% at 12 weeks for the control group participant (male, 35 years old).



**Table 1** Baseline characteristics of the study population

Characteristics	Flaxseed group ( <i>n</i> = 25)	Control group ( <i>n</i> = 25)	<i>p</i> -value
Age, year	35.44 ± 10.85	36.32 ± 10.00	0.767
Sex			0.395
Male, <i>n</i> (%)	13 (52.0)	10 (40.0)	
Female, <i>n</i> (%)	12 (48.0)	15 (60.0)	
Occupation			0.658
Professionals/technicians, <i>n</i> (%)	13 (52.0)	13 (52.0)	
Sales/workers/farmers, <i>n</i> (%)	8 (32.0)	6 (24.0)	
Other, <i>n</i> (%)	4 (16.0)	6 (24.0)	
Educational attainment			0.768
Primary school and below, <i>n</i> (%)	2 (8.0)	1 (4.0)	
Junior and senior high school, <i>n</i> (%)	6 (24.0)	8 (32.0)	
College and above, <i>n</i> (%)	17 (68.0)	16 (64.0)	
Anthropometry			
Height, cm	168.59 ± 7.43	166.33 ± 9.69	0.358
Weight, kg	90.5 ± 12.1	87.4 ± 15.2	0.440
BMI, kg m <sup>-2</sup>	31.33 (29.50, 34.40)	31.13 (28.39, 33.34)	0.550
Body fat, %	38.02 ± 5.10	37.53 ± 4.73	0.727
Obesity degree, %	152.70 (143.90, 161.70)	147.70 (142.10, 157.40)	0.105
WHR	0.91 ± 0.07	0.94 ± 0.05	0.119
VFA, cm <sup>2</sup>	199.20 ± 72.86	172.62 ± 60.06	0.166
Skeletal muscle mass, kg	30.96 ± 5.60	30.44 ± 5.78	0.748
Daily dietary intake			
Energy, kcal d <sup>-1</sup>	2157.36 ± 909.00	2044.08 ± 828.72	0.647
Protein, g d <sup>-1</sup>	75.30 (58.60, 101.70)	65.70 (54.10, 86.80)	0.105
Fat, g d <sup>-1</sup>	57.80 (31.90, 85.40)	53.70 (35.40, 83.20)	0.816
Carbohydrates, g d <sup>-1</sup>	299.81 ± 142.41	303.68 ± 132.90	0.921
Dietary fiber, g d <sup>-1</sup>	9.90 ± 5.35	11.01 ± 5.93	0.491
Vitamin B <sub>1</sub> , mg d <sup>-1</sup>	1.10 (0.70, 1.70)	1.22 (0.90, 1.80)	0.547
Vitamin B <sub>2</sub> , mg d <sup>-1</sup>	0.88 (0.50, 1.90)	1.12 (0.50, 1.90)	0.992
Vitamin B <sub>3</sub> , mg d <sup>-1</sup>	12.68 ± 9.64	16.05 ± 11.37	0.263
Vitamin C, mg d <sup>-1</sup>	66.85 (14.60, 139.40)	49.10 (16.30, 89.90)	0.621
Vitamin E, mg d <sup>-1</sup>	9.98 (3.40, 19.10)	9.83 (6.80, 17.80)	0.869
Zinc, mg d <sup>-1</sup>	8.60 ± 5.55	10.87 ± 7.75	0.240
Selenium, mg d <sup>-1</sup>	40.41 (13.70, 69.20)	41.70 (19.60, 79.20)	0.669
Physical activity, MET-min per week	2478 (1409, 3943)	2573 (1517, 4172)	0.648
MRI-PDFF (%)	10.80 (9.10, 18.63)	10.30 (7.64, 14.10)	0.159
Liver function			
TBIL (μmol L <sup>-1</sup> )	14.98 (10.60, 16.90)	12.33 (10.20, 15.20)	0.204
DBIL (μmol L <sup>-1</sup> )	3.94 (3.20, 5.50)	3.42 (2.80, 4.70)	0.09
IBIL (μmol L <sup>-1</sup> )	10.68 (7.60, 12.40)	8.38 (6.10, 10.10)	0.055
ALT (U L <sup>-1</sup> )	40.00 (34.00, 57.00)	34.00 (26.00, 52.50)	0.171
AST (U L <sup>-1</sup> )	23.00 (21.50, 28.00)	22.00 (19.00, 27.00)	0.173
GGT (U L <sup>-1</sup> )	35.00 (30.50, 54.00)	30.00 (23.50, 40.00)	0.109
CHE (U L <sup>-1</sup> )	11 205.48 ± 1142.70	10 680.44 ± 1103.55	0.105
Blood lipids			
TC (mmol L <sup>-1</sup> )	5.22 ± 0.80	5.05 ± 0.65	0.422
TG (mmol L <sup>-1</sup> )	2.11 ± 0.74	2.69 ± 1.65	0.120
HDL-C (mmol L <sup>-1</sup> )	1.01 ± 0.09	0.98 ± 0.12	0.307
LDL-C (mmol L <sup>-1</sup> )	3.43 ± 0.76	3.21 ± 0.53	0.243
Apo A1 (mmol L <sup>-1</sup> )	0.94 ± 0.09	0.93 ± 0.09	0.621
Apo B (mmol L <sup>-1</sup> )	0.94 ± 0.01	0.91 ± 0.14	0.463
LP(a) (mmol L <sup>-1</sup> )	93.50 (45.50, 236.40)	133.80 (58.30, 211.30)	0.491
Glycemic control parameters			
GLU (mmol L <sup>-1</sup> )	5.06 ± 0.56	5.40 ± 0.62	0.463
FINS (pmol L <sup>-1</sup> )	113.36 (92.70, 136.60)	114.70 (93.50, 127.70)	0.977

Abbreviations: BMI, body mass index; WHR, waist to hip ratio; VFA, visceral fat area; MET, metabolic equivalent; MRI-PDFF, magnetic resonance imaging proton density fat fraction; TBIL, total bilirubin; DBIL, direct bilirubin; IBIL, indirect bilirubin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transpeptidase; CHE, cholinesterase; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Apo A1, apolipoprotein A1; Apo B, apolipoprotein B; LP(a), lipoprotein (a); GLU, glucose; FINS, fasting insulin. Values are displayed as *n* (%), mean ± SD, or median (IQR).

### Effects of flaxseed powder consumption on the relative abundance of the gut microbiota

To explore the potential effects of flaxseed powder on the gut microbiota of NAFLD patients, 16S rRNA gene sequencing was

performed on the feces of all participants. Fig. 4A showed the baseline differences in the gut microbiota distribution at the phylum level between the two groups. The results indicated that there was no statistically significant difference in the distribution of major phyla between the intervention group and



**Table 2** Daily dietary intake and physical activity of subjects during the 12-week intervention

Characteristics	Flaxseed group (n = 25)			Control group (n = 25)		
	Baseline (n = 25)	Week 12 (n = 25)	<i>p</i> <sup>a</sup>	Baseline (n = 25)	Week 12 (n = 25)	<i>p</i> <sup>b</sup>
Energy, kcal d <sup>-1</sup>	2157.36 ± 909.00	2023 ± 845.67	0.878	2044.08 ± 828.72	2154.08 ± 749.67	0.539
Protein, g d <sup>-1</sup>	75.30 (58.60, 101.70)	79.20 (61.40, 98.30)	0.763	65.70 (54.10, 86.80)	61.83 (49.64, 88.27)	0.227
Fat, g d <sup>-1</sup>	57.80 (31.90, 85.40)	54.20 (36.70, 89.30)	0.698	53.70 (35.40, 83.20)	59.6 (46.80, 80.60)	0.816
Carbohydrates, g d <sup>-1</sup>	299.81 ± 142.41	276.56 ± 126.89	0.425	303.68 ± 132.90	274.39 ± 118.60	0.434
Dietary fiber, g d <sup>-1</sup>	9.90 ± 5.35	10.3 ± 4.89	0.673	11.01 ± 5.93	10.82 ± 3.67	0.731
Vitamin B <sub>1</sub> , mg d <sup>-1</sup>	1.10 (0.70, 1.70)	1.13 (0.80, 1.80)	0.621	1.22 (0.90, 1.80)	1.05 (0.80, 1.60)	0.628
Vitamin B <sub>2</sub> , mg d <sup>-1</sup>	0.88 (0.50, 1.90)	0.92 (0.48, 1.93)	0.342	1.12 (0.50, 1.90)	1.09 (0.60, 1.85)	0.870
Vitamin B <sub>3</sub> , mg d <sup>-1</sup>	12.68 ± 9.64	11.99 ± 8.63	0.385	16.05 ± 11.37	15.62 ± 9.73	0.473
Vitamin C, mg d <sup>-1</sup>	66.85 (14.60, 139.40)	68.43 (25.29, 125.36)	0.632	49.10 (16.30, 89.90)	43.50 (14.62, 76.40)	0.384
Vitamin E, mg d <sup>-1</sup>	9.98 (3.40, 19.10)	10.04 (4.21, 19.37)	0.531	9.83 (6.80, 17.80)	8.79 (5.30, 17.6.40)	0.737
Zinc, mg d <sup>-1</sup>	8.60 ± 5.55	8.40 ± 5.02	0.872	10.87 ± 7.75	9.93 ± 7.45	0.390
Selenium, mg d <sup>-1</sup>	40.41 (13.70, 69.20)	42.32 (14.78, 62.73)	0.691	41.70 (19.60, 79.20)	43.10 (21.50, 68.60)	0.683
Physical activity, MET-min per week	2478 (1409, 3943)	2583 (1672, 3856)	0.839	2573 (1517, 4172)	2450 (1493, 4078)	0.721

Abbreviation: MET, metabolic equivalent. Values are displayed as mean ± SD, or median (IQR). <sup>a,b</sup>*p* values indicate differences between the control and treatment groups at the baseline and 12 weeks.

**Table 3** Anthropometric data of the subjects during the trial

Characteristics	Flaxseed group			Control group		
	Baseline (n = 25)	Week 12 (n = 25)	Change	Baseline (n = 25)	Week 12 (n = 25)	Change
Weight, kg	90.5 ± 12.1	88.1 ± 10.6	-2.4 ± 2.3	87.4 ± 15.2	87.5 ± 14.7	0.1 ± 2.9 <sup>c</sup>
BMI, kg m <sup>-2</sup>	31.33 (29.50, 34.40)	30.67 (28.72, 32.62)	-0.92 (-1.36, -0.23)	31.13 (28.39, 33.34)	31.13 (28.39, 33.34)	0.02 (-0.43, 0.70) <sup>c</sup>
Body fat, %	38.02 ± 5.10	31.62 ± 3.62 <sup>a</sup>	-6.40 ± 2.16	37.53 ± 4.73	36.22 ± 5.33 <sup>b</sup>	-1.30 ± 1.12 <sup>c</sup>
Obesity degree, %	152.70 (143.90, 161.70)	143.40 (130.10, 151.80) <sup>a</sup>	-12.80 (-15.70, -11.70)	147.70 (142.10, 157.40)	142.80 (134.10, 153.50)	-5.30 (-23.40, 9.80)
WHR	0.91 ± 0.07	0.89 ± 0.04	-0.02 ± 0.08	0.94 ± 0.05	0.93 ± 0.03 <sup>b</sup>	-0.01 ± 0.06
VFA, cm <sup>2</sup>	199.20 ± 72.86	156.20 ± 46.75 <sup>a</sup>	-28.90 (-41.70, -21.40)	172.62 ± 60.06	168.90 ± 63.73	-11.30 (-26.10, 48.90) <sup>c</sup>
Skeletal muscle mass, kg	30.96 ± 5.60	31.81 ± 5.76	0.90 (0.30, 1.40)	30.44 ± 5.78	31.48 ± 6.79	1.10 (-0.10, 2.20)

Abbreviations: BMI, body mass index; WHR, waist to hip ratio; VFA, visceral fat area. Values are displayed as *n* (%), mean ± SD, or median (IQR). <sup>a</sup> indicates a statistically significant difference between the baseline and week 12 in the flaxseed group or the control group, *p* < 0.05; <sup>b</sup> indicates a statistically significant difference between the flaxseed group and the control group at week 12, *p* < 0.05; <sup>c</sup> indicates a statistically significant difference between the change from the baseline to week 12 in the flaxseed group and the control group, *p* < 0.05.

the control group at the baseline. After 12 weeks of intervention, the relative abundance of *Bacteroidetes* and *Actinobacteria* in the gut microbiota of the intervention group was significantly higher than that of the control group, while the relative abundance of *Firmicutes* in the intervention group was significantly lower than that of the control group (*P* < 0.05) (Fig. 4B). Fig. 5A shows the baseline differences in the gut microbiota distribution at the genus level between the two groups. The results showed no statistically significant difference in the distribution of gut microbiota at the genus level between the two groups at the baseline. Fig. 5B shows the genera with different distributions at the genus level of the gut microbiota between the two groups at 12 weeks. After 12 weeks of flaxseed powder intervention, the relative abundance of *Clostridium\_sensu\_stricto\_1*, *Parasutterella*, *Lachnospiraceae\_NK4A136\_group*, *Eubacterium\_xylanophilum\_group*, and *Bifidobacterium* in the gut microbiota of the intervention group was significantly higher than that of the control group, while

the relative abundance of *Coriobacteriaceae\_UCG-002* was significantly lower than that of the control group (*P* < 0.05) (Fig. 5B).

#### Analysis of the correlation between the gut microbiota and glucose–lipid metabolism and liver function in patients with NAFLD

To further investigate whether the improvement of glycolipid metabolism and liver function indicators in NAFLD patients was related to changes in the gut microbiota, the Spearman method was used to perform correlation analysis on the glycolipid metabolism, liver function indicators, and gut microbiota with different abundances in NAFLD patients at 12 weeks, as shown in Fig. 6. The analysis revealed that *Clostridium\_sensu\_stricto\_1*, *Lachnospiraceae\_NK4A136\_group*, and *Bifidobacterium* were significantly correlated with numerous indicators of glycolipid metabolism and liver function (*P* < 0.05). Among them, *Bifidobacterium* was significantly positively





Table 4 Biochemical data of the subjects during the trial

Characteristics	Flaxseed group			Control group		
	Baseline (n = 25)	Week 12 (n = 25)	Change	Baseline (n = 25)	Week 12 (n = 25)	Change
<b>Liver function</b>						
TBIL ( $\mu\text{mol L}^{-1}$ )	14.98 (10.60, 16.90)	9.71 (7.80, 12.60) <sup>a</sup>	-4.16 (-5.30, -2.90)	12.33 (10.20, 15.20)	9.13 (5.80, 14.10) <sup>a</sup>	-3.21 (-4.10, -2.40) <sup>c</sup>
DBIL ( $\mu\text{mol L}^{-1}$ )	3.94 (3.20, 5.50)	3.29 (2.30, 3.70) <sup>a</sup>	-1.03 (-1.80, -0.80)	3.42 (2.80, 4.70)	2.44 (1.90, 4.00) <sup>a</sup>	-0.80 (-1.00, -0.50) <sup>c</sup>
IBIL ( $\mu\text{mol L}^{-1}$ )	10.68 (7.60, 12.40)	6.80 (5.70, 8.40) <sup>a</sup>	-3.29 $\pm$ 1.29	8.38 (6.10, 10.10)	6.69 (4.00, 10.1)	-1.78 $\pm$ 2.13 <sup>c</sup>
ALT (U L <sup>-1</sup> )	40.00 (34.00, 57.00)	37.00 (28.00, 49.00)	-6.00 (-12.00, -3.00)	34.00 (26.00, 52.50)	37.00 (21.50, 42.50)	-4.00 (-12.00, -2.00)
AST (U L <sup>-1</sup> )	23.00 (21.50, 28.00)	18.00 (16.00, 23.00) <sup>a</sup>	-6.00 (-6.00, -4.00)	22.00 (19.00, 27.00)	20.00 (18.00, 26.00)	-1.00 (-2.50, -1.00) <sup>c</sup>
GGT (U L <sup>-1</sup> )	35.00 (30.50, 54.00)	36.00 (22.50, 54.50)	-8.00 (-10.00, 0.50)	30.00 (23.50, 40.00)	24.00 (16.50, 41.50)	-6.00 (-7.00, -4.00)
CHE (U L <sup>-1</sup> )	11 205.48 $\pm$ 1142.70	10 827.76 $\pm$ 2145.83	377.72 $\pm$ 1725.43	10 680.44 $\pm$ 1103.55	9948.28 $\pm$ 1969.47	732.16 $\pm$ 998.36
<b>Blood lipids</b>						
TC (mmol L <sup>-1</sup> )	5.22 $\pm$ 0.80	4.75 $\pm$ 0.78 <sup>a</sup>	-0.34 (-0.50, -0.20)	5.05 $\pm$ 0.65	4.73 $\pm$ 0.70	-0.45 (-0.50, -0.40)
TG (mmol L <sup>-1</sup> )	2.11 $\pm$ 0.74	1.71 $\pm$ 0.63 <sup>a</sup>	-0.40 $\pm$ 0.36	2.69 $\pm$ 1.65	2.65 $\pm$ 1.52 <sup>b</sup>	-0.04 $\pm$ 0.60 <sup>c</sup>
HDL-C (mmol L <sup>-1</sup> )	1.01 $\pm$ 0.09	1.12 $\pm$ 0.17 <sup>a</sup>	0.10 $\pm$ 0.08	0.98 $\pm$ 0.12	0.98 $\pm$ 0.12 <sup>b</sup>	0.00 $\pm$ 0.06 <sup>c</sup>
LDL-C (mmol L <sup>-1</sup> )	3.43 $\pm$ 0.76	3.20 $\pm$ 0.98	-0.23 $\pm$ 0.28	3.21 $\pm$ 0.53	3.16 $\pm$ 0.52	-0.05 $\pm$ 0.14 <sup>c</sup>
Apo A1 (mmol L <sup>-1</sup> )	0.94 $\pm$ 0.09	1.06 $\pm$ 0.13 <sup>a</sup>	0.12 $\pm$ 0.04	0.93 $\pm$ 0.09	0.94 $\pm$ 0.05 <sup>b</sup>	0.01 $\pm$ 0.05 <sup>c</sup>
Apo B (mmol L <sup>-1</sup> )	0.94 $\pm$ 0.01	0.88 $\pm$ 0.26	-0.06 $\pm$ 0.08	0.91 $\pm$ 0.14	0.90 $\pm$ 0.11	-0.01 $\pm$ 0.15
LP(a) (mmol L <sup>-1</sup> )	93.50 (45.50, 236.40)	48.70 (23.20, 187.30)	-22.50 (-44.00, -18.40)	133.80 (58.30, 211.30)	87.50 (48.00, 157.60)	-38.80 (-59.00, -14.80)
<b>Glycemic control parameters</b>						
GLU (mmol L <sup>-1</sup> )	5.06 $\pm$ 0.56	5.10 $\pm$ 0.49	0.03 $\pm$ 0.56	5.40 $\pm$ 0.62	5.25 $\pm$ 0.83	-0.15 $\pm$ 1.03
FINS (pmol L <sup>-1</sup> )	113.36 (92.70, 136.60)	106.86 (95.30, 146.40)	7.42 (-29.70, 43.80)	114.70 (93.50, 127.70)	105.03 (90.70, 159.00)	10.96 (-30.40, 51.90)

Abbreviations: TBIL, total bilirubin; DBIL, direct bilirubin; IBIL, indirect bilirubin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transpeptidase; CHE, cholinesterase; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Apo A1, apolipoprotein A1; Apo B, apolipoprotein B; LP(a), lipoprotein (a); GLU, glucose; FINS, fasting insulin. Values are displayed as *n* (%), mean  $\pm$  SD, or median (IQR). <sup>a</sup> indicates a statistically significant difference between the baseline and week 12 in the flaxseed group or the control group, *p* < 0.05; <sup>b</sup> indicates a statistically significant difference between the flaxseed group and the control group at week 12, *p* < 0.05; <sup>c</sup> indicates a statistically significant difference between the change from the baseline to week 12 in the flaxseed group and the control group, *p* < 0.05.



Table 5 Liver fat content data of the subjects during the trial

Characteristics	Flaxseed group			Control group		
	Baseline	Week 12	Change	Baseline	Week 12	Change
MRI-PDFF (%)	10.80 (9.10, 18.63) (n = 25)	8.17 (6.70, 11.22) <sup>a</sup> (n = 25)	-5.28 (-8.77, -3.30)	10.30 (7.64, 14.10) (n = 25)	8.85 (5.09, 15.41) (n = 25)	-1.23 (-2.31, 0.51) <sup>c</sup>
MRI-PDFF (5%–10%)	9.10 (8.96, 9.20) (n = 12)	6.65 (5.17, 11.71) (n = 12)	-2.91 (-3.80, -2.57)	7.64 (6.08, 9.50) (n = 13)	5.25 (9.10, 7.47) (n = 13)	-0.11 (-2.29, -0.43)
MRI-PDFF (10%–25%)	18.62 (16.52, 20.20) (n = 13)	9.66 (7.65, 10.65) <sup>a</sup> (n = 13)	-8.87 (-9.55, -7.59)	15.71 (12.40, 19.62) (n = 12)	13.44 (11.13, 18.45) <sup>b</sup> (n = 12)	-1.52 (-3.12, 1.06) <sup>c</sup>

Abbreviation: MRI-PDFF, magnetic resonance imaging proton density fat fraction. Values are displayed as median (IQR). <sup>a</sup> indicates a statistically significant difference between the baseline and week 12 in the flaxseed group or the control group,  $p < 0.05$ ; <sup>b</sup> indicates a statistically significant difference between the flaxseed group and the control group at week 12,  $p < 0.05$ ; <sup>c</sup> indicates a statistically significant difference between the change from the baseline to week 12 in the flaxseed group and the control group,  $p < 0.05$ .

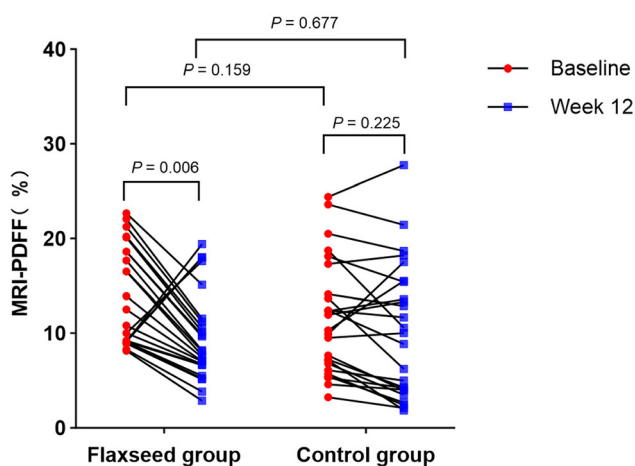


Fig. 2 The liver fat content of each subject at the baseline and 12 weeks.

correlated with serum Apo A1 and HDL-C levels, and significantly negatively correlated with AST, TBIL, DBIL, I-BIL, LDL-C, lipoprotein (a) (LP(a)), TC, and TG levels ( $P < 0.05$ ). *Clostridium\_sensu\_stricto\_1* was negatively correlated with glucose ( $P < 0.05$ ). *Coriobacteriaceae\_UCG-002* was significantly positively correlated with serum ALT, AST, DBIL, and TG levels ( $P < 0.05$ ). However, the *Lachnospiraceae\_NK4A136\_group* showed a significant negative correlation with serum AST and TG levels ( $P < 0.05$ ).

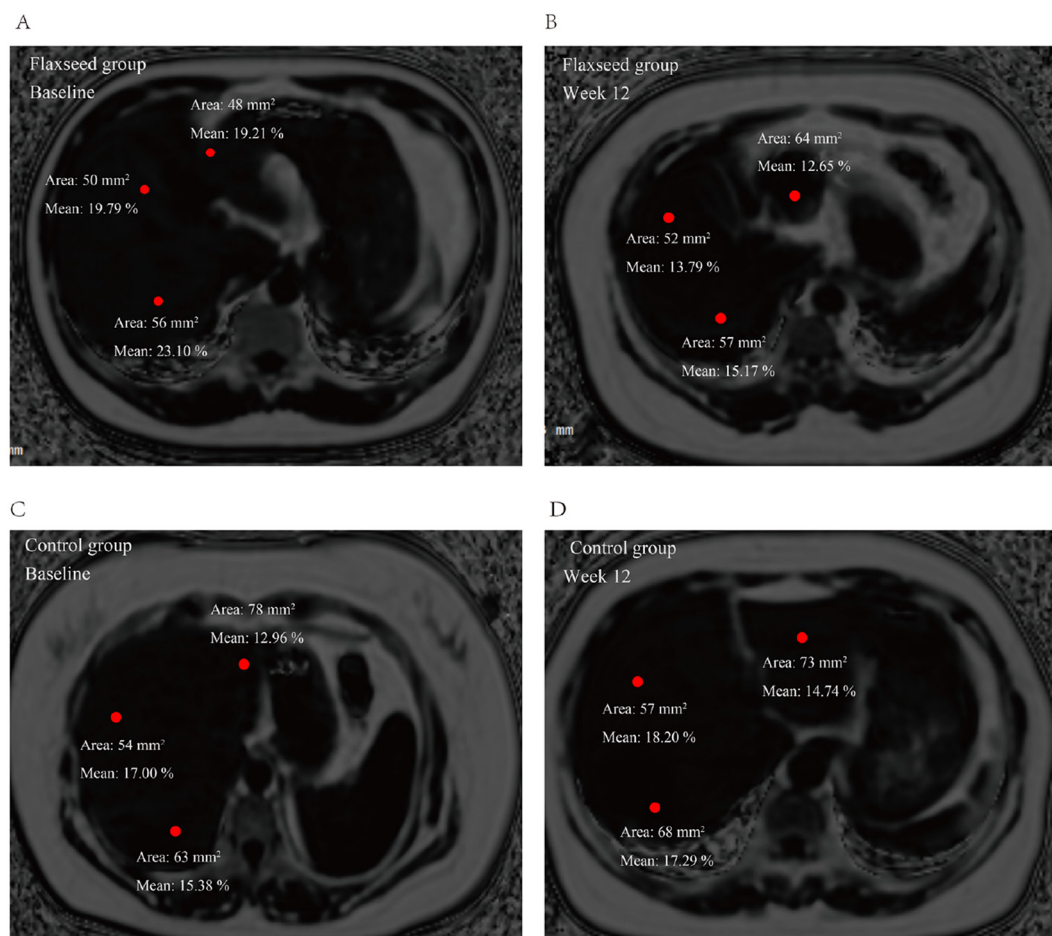
## Discussion

This randomized controlled clinical trial aimed to explore the effects of flaxseed powder intervention on improving NAFLD patients' conditions. Results indicated that taking 30 g of flaxseed powder daily for 12 weeks could reduce liver fat content, obesity and visceral fat area, improve liver function and blood lipid profile, and increase the abundance of gut microbiota species in NAFLD patients. Previous clinical studies have shown that flaxseed powder has a significant improvement effect on NAFLD patients,<sup>32–34</sup> consistent with our findings.

Currently, liver biopsy is the gold standard for evaluating liver histopathology in patients with NAFLD. However, this invasive technique might cause complications associated with the procedure. Therefore, it is not practical to use it to quantify the dynamic changes in fatty liver.<sup>35</sup> In this study, MRI-PDFF was used to measure the liver fat content. MRI-PDFF is the most reliable non-invasive method for detecting steatosis, and due to its feasibility and accuracy, it has been adopted as a primary outcome indicator in multiple clinical trials.<sup>36</sup> The results of this study indicated that after 12 weeks of intervention, the liver fat content in the flaxseed intervention group showed a significant reduction compared to the baseline, while no such change was observed in the control group. Interestingly, further subgroup analysis revealed that flaxseed intervention significantly reduced the liver fat content in patients with moderate NAFLD, whereas in the mild NAFLD subgroup, there was no statistically significant difference in the liver fat content before and after the intervention. In an animal study conducted by Chao Yang *et al.*,<sup>33</sup> it was found that flaxseed powder significantly improved hepatic steatosis in NAFLD mice, with effects being dose-dependent – the higher the flaxseed powder dose, the more evident the improvement. In two other randomized, controlled, clinical trials, both of which used 30 g of whole flaxseed powder for 12 weeks of intervention in patients with NAFLD, liver steatosis and fibrosis were assessed using transient elastography in all patients, and the results indicated significant improvement in both fibrosis scores and the percentage of steatosis in the flaxseed intervention group.<sup>29,37</sup> However, both studies did not perform a stratified analysis, so it remained unclear whether flaxseed powder interventions had different effects on mild NAFLD versus moderate NAFLD. Additionally, there has been limited prior research on related populations; thus, this study also offers a new perspective for future research on using flaxseed powder to improve the condition of NAFLD patients.

In the current study, a 12-week intervention with flaxseed powder led to a significant reduction in body weight, BMI, body fat percentage, and visceral fat area in patients with NAFLD. Body composition indicators were associated with the risk and severity of NAFLD. NAFLD patients with a BMI greater than 25 kg m<sup>-2</sup> had a higher risk of liver fibrosis compared to





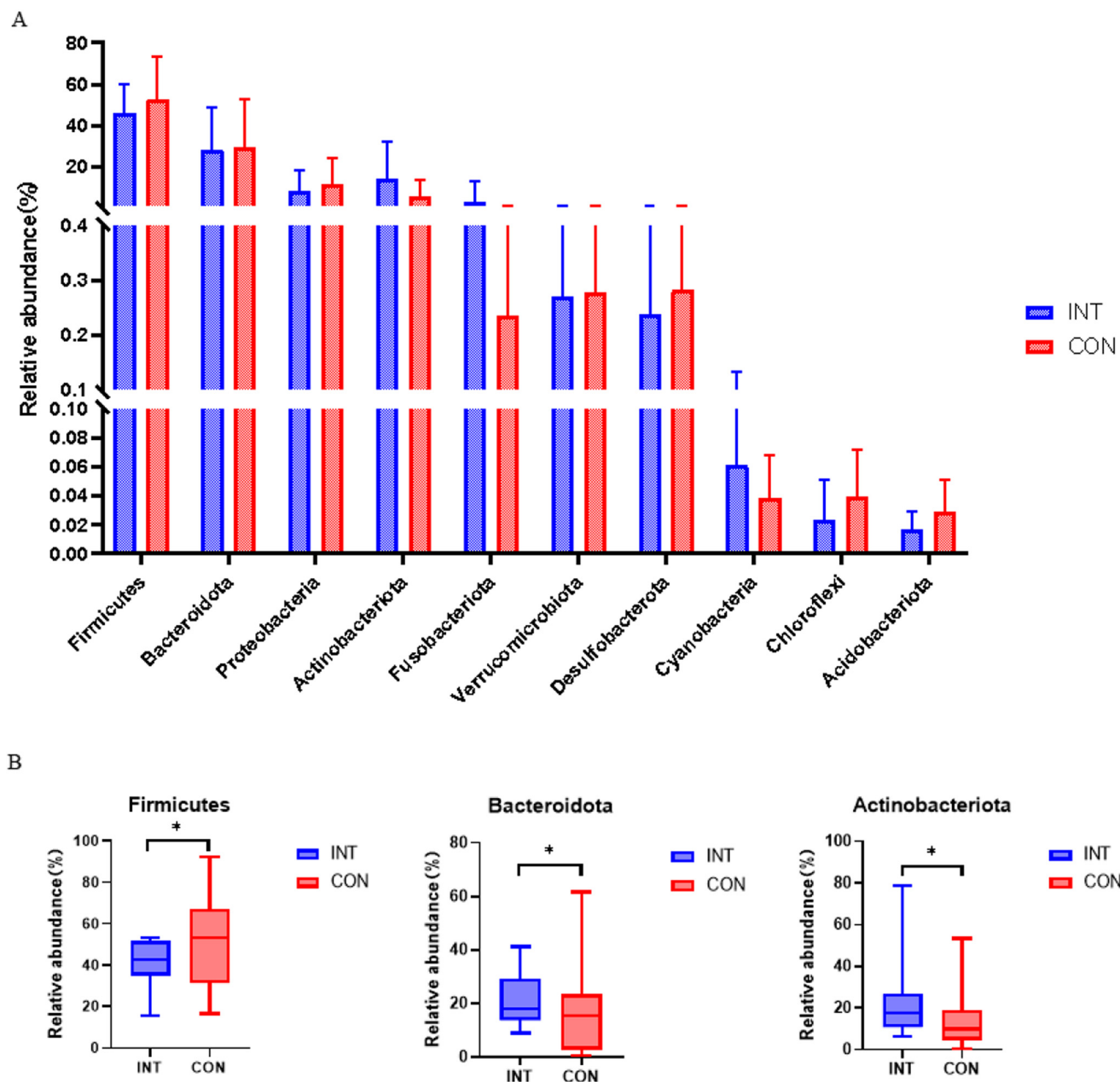
**Fig. 3** Levels at the baseline (A) and 12 weeks (B) for 1 subject in the flaxseed group and at the baseline (C) and 12 weeks (D) for 1 subject in the control group. The liver fat content values for each subject were based on the average lipid content of 9 liver segments shown within a circular region of approximately 2 cm in diameter.

those with normal weight, and the reduction of body weight delayed the progression of NAFLD,<sup>38,39</sup> and body fat percentage was highly sensitive in predicting NAFLD risk.<sup>40</sup> Previously, flaxseed has been widely used for weight loss among individuals who are overweight or obese. Research has shown that flax lignans can help prevent obesity or aid in weight loss by regulating adiponectin levels and up-regulating the level of fat oxidation in skeletal muscles. In addition, flax lignans could reduce the synthesis levels of sterol regulatory elements necessary for TG production and increase short-chain fatty acid concentrations in the intestines, thereby enhancing satiety to promote weight loss.<sup>41</sup> Meanwhile, the soluble fiber in flaxseed dietary fiber has also been shown to suppress appetite and delay gastric emptying by absorbing water and swelling to exert a weight loss effect.<sup>42</sup> A three-arm randomized controlled trial conducted by Bongartz *et al.*<sup>43</sup> also demonstrated that flaxseed soluble dietary fiber supplementation reduced body weight and waist-hip circumference in overweight and obese patients. Meanwhile, the high concentration of ALA contained in flaxseed also played an important role in the weight reduction effect. ALA was converted into

eicosapentaenoic acid and docosahexaenoic acid when it entered the body, thus exerting a weight reduction effect.<sup>44</sup> Finally, flaxseed dietary fiber altered the composition of the gut microbiota, thereby accelerating intestinal absorption of lignans and promoting the formation of lignan metabolite enterolactone, which was negatively correlated with weight gain.<sup>45</sup> This also reflected the superiority of flaxseed powder supplementation compared to supplementation with individual flaxseed components.

Flaxseed is considered natural and effective in improving liver function in NAFLD patients.<sup>46</sup> Serum bilirubin levels were used to assess the ability of hepatocytes to process bilirubin. Elevated serum TBIL levels should be divided into direct and indirect bilirubin components. Elevated DBIL (conjugated bilirubin) was indicative of hepatocyte dysfunction or cholestasis, whereas damage to hepatocytes led to a decrease in the liver's ability to process bilirubin, which could lead to an elevation of serum bilirubin levels.<sup>47</sup> Asymptomatic elevation of liver enzymes was a possible clinical manifestation of NAFLD. Among these, ALT showed the strongest correlation with fat accumulation in the liver and was most commonly

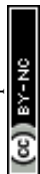




**Fig. 4** Comparison of the intestinal flora structure at the phylum level between the groups at the baseline (A) and comparison of the intestinal flora structure at the phylum level between the groups at 12 weeks (B). \* indicates a statistically significant difference,  $P < 0.05$ .

found in the cytoplasm of hepatocytes. Serum ALT has historically served as an indicator of inflammation from hepatocellular injury in diseases such as cirrhosis, hepatitis, hepatocellular carcinoma, and alcoholic liver disease.<sup>48–50</sup> Since ALT is a sensitive indicator for recognizing NAFLD injury, it was frequently elevated when liver injury was moderate.<sup>51,52</sup> In individuals with NAFLD, ALT was usually greater than AST and  $AST/ALT < 1$  was typical. The cytoplasm and mitochondria of cells contained AST, and as the disease progressed, hepatocytes released more AST than ALT because the mitochondria of the cells were damaged, and the elevation of ALT and AST varied with the extent and duration of liver disease.<sup>53,54</sup> The

results of this study showed that the serum TBIL, DBIL, IBIL and AST levels of NAFLD patients in the intervention group at the end of the intervention were significantly lower than those before the intervention, and the serum TBIL and DBIL levels of the control group also improved at the end of the intervention, but the improvement was not as significant as that of the intervention group, which suggested that flaxseed has the ability to ameliorate the role of liver injury in NAFLD patients. Previous studies have reached similar conclusions. In a 12-week randomized controlled trial conducted by Yari *et al.*<sup>29</sup> with 50 NAFLD patients, the intervention group consumed 30 g of flaxseed powder daily. Both the intervention and



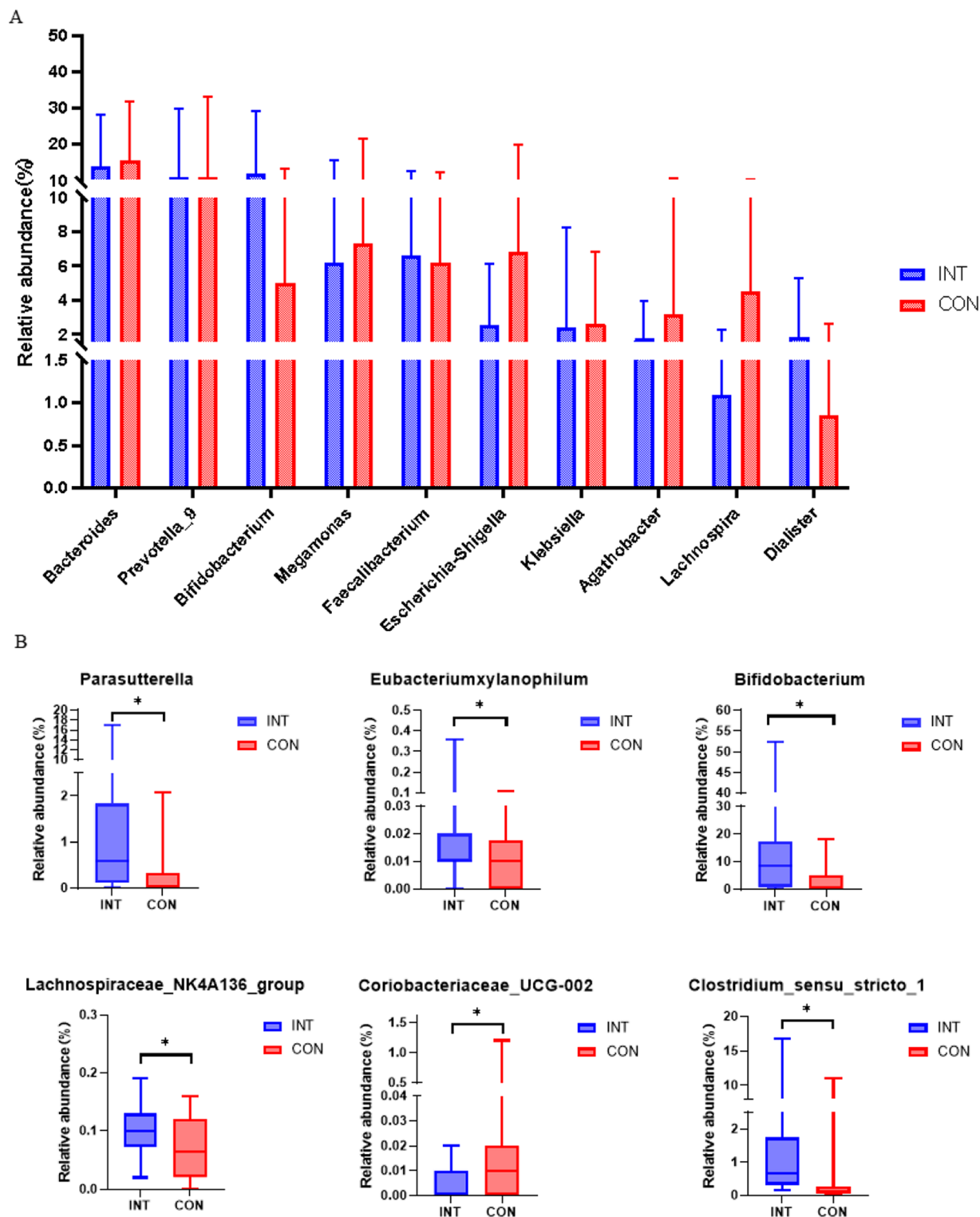
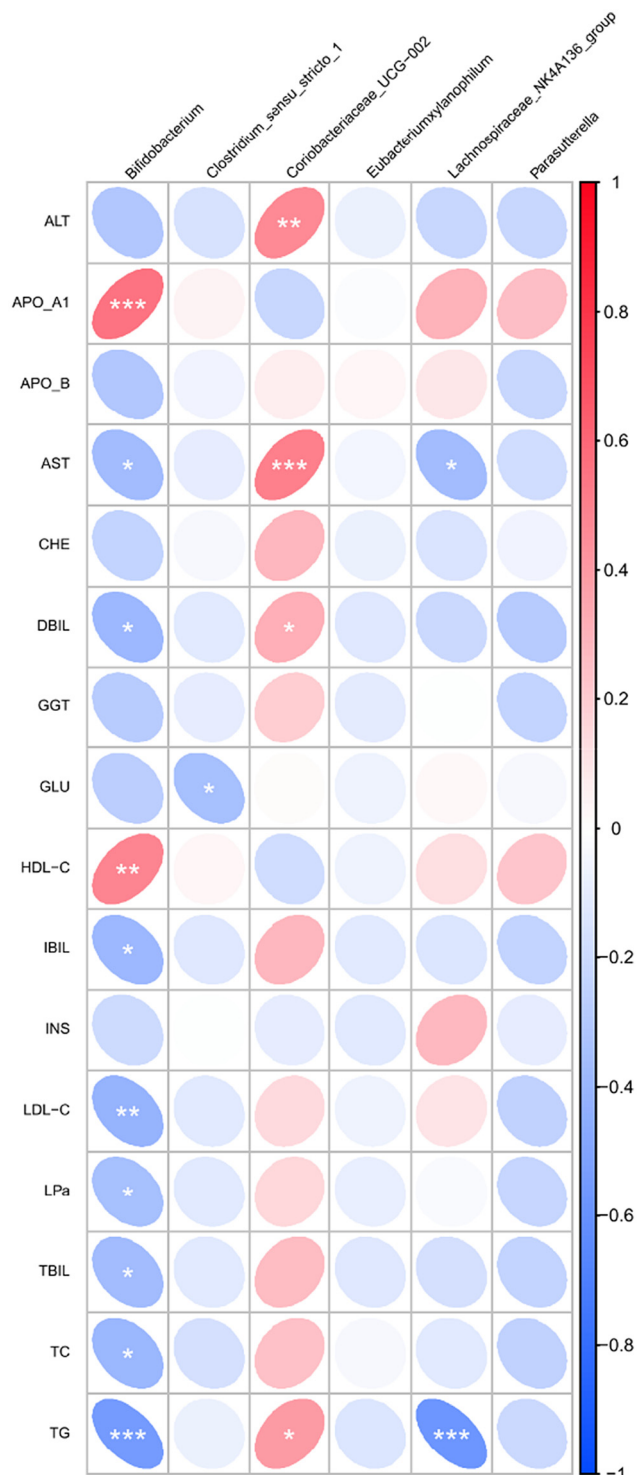


Fig. 5 Comparison of the intestinal flora structure at the genus level between the groups at the baseline (A) and comparison of the intestinal flora structure at the genus level between the groups at 12 weeks (B). \* indicates a statistically significant difference,  $P < 0.05$ .

control groups also received health education. The results showed improvement in the liver fat content and liver function indicators in both groups, with the intervention group showing more significant liver function improvement. In

addition, Davis *et al.*<sup>55</sup> investigated the ameliorative effect of flaxseed meal on fatty liver in NAFLD model laying hens, and the results showed that defatted flaxseed meal and whole flaxseed meal could significantly reduce the serum level of AST in



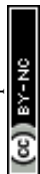


**Fig. 6** Heat map of the Spearman correlation coefficients between the differential microbiota and the indicators of glucose–lipid metabolism and liver function. Colors indicate positive or negative correlation coefficients; red indicates a positive correlation, blue indicates a negative correlation, and darker color means a stronger correlation; \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ .

NAFLD laying hens, and at the same time, the whole flaxseed meal was able to reduce hepatocyte swelling, which indicated that flaxseed could effectively improve hepatocyte injury and restore liver function.

In addition, the results of this study showed that after 90 days of flaxseed powder intervention in patients with NAFLD, the levels of TG decreased and the levels of HDL-C and Apo A1 increased in the lipid indexes of the intervention population. HDL-C had the role of reverse cholesterol, and Apo A1 was an important component of HDL-C, and the increase in the levels of HDL-C and Apo A1 and the decrease in the levels of TG and LDL-C indicated the restoration of lipid homeostasis.<sup>56</sup> This result indicated that flaxseed powder had an improving effect on lipid metabolism disorders in NAFLD patients, which was consistent with the results of previous studies. Yang *et al.* investigated the effect of flaxseed on high-fat diet-induced NAFLD model mice, and intervened with the addition of flaxseed at the doses of 10 g, 20 g, and 30 g of flaxseed per 100 g of feed, which showed that the effect of flaxseed on the NAFLD model mice at the doses of 10 g per 100 g and 20 g per 100 g flaxseed intervention significantly reduced serum TG and LDL-C levels in NAFLD model mice.<sup>33</sup> In addition, a flaxseed powder intervention study conducted on 50 patients with dyslipidemia found that taking 30 g of flaxseed powder daily for 90 days effectively increased serum HDL-C levels, reduced TC levels, and lowered the risk of atherosclerosis.<sup>57</sup> Flaxseed is rich in n-3 PUFA, and has been shown to be used to ameliorate NAFLD; it could reduce fatty acid and cholesterol biosynthesis by decreasing the expression of lipogenic genes and decreasing the number of lipid synthesizing transcription factors.<sup>58</sup> For many years, the physiological activities of lignans, especially their antioxidant properties, has been extensively studied. The antioxidant activity of flaxseed lignans, expressed through their metabolites, could effectively lower serum cholesterol and reduce the development of atherosclerosis.<sup>59</sup> Furthermore, a high intake of flax lignans could also reduce serum TC.<sup>41</sup> A double-blind, randomized crossover trial conducted by Kristensen *et al.* examined the lipid metabolism improvement effects of flaxseed dietary fiber. The results showed that supplementing with flaxseed dietary fiber reduced serum TC levels by 12% and LDL-C levels by 15%.<sup>60</sup> Therefore, the lipid metabolism improvement effect of flaxseed might be partially attributed to its high n-3 PUFA content, lignans, and flaxseed dietary fiber. In addition, a meta-analysis showed that whole flaxseed was more effective in improving lipid metabolism compared to a single component. This also suggested a synergistic effect between the biological functions of different components of flaxseed.<sup>61</sup>

Previous studies have shown that multiple alpha diversity indices of the gut microbiota are decreased and species richness is significantly reduced in patients with NAFLD compared to healthy populations.<sup>62</sup> The results of this study indicated that daily consumption of 30 g of flaxseed powder significantly



improved the species richness of the gut microbiota in patients with NAFLD, with notable increases in the Shannon and Simpson indices, leading to enhanced diversity of the gut microbiome. At the phylum level, the relative abundance changes of *Firmicutes* and *Bacteroidetes* were the primary differences in the gut microbiota between NAFLD patients and healthy individuals; the abundance of *Firmicutes* significantly increased in NAFLD patients, while the abundance of *Bacteroidetes* significantly decreased, resulting in a significantly higher *Firmicutes/Bacteroidetes* (F/B) ratio.<sup>63</sup> The increase in the F/B ratio indicated an increase in the overall fermentation activity of the microbiota, leading to increased efficiency of energy absorption in the intestine.<sup>64</sup> The F/B ratio was positively correlated with obesity and BMI.<sup>65</sup> In the results of the present study, the abundance of *Firmicutes* in the gut microbiota of the intervention group was significantly lower than that of the control group, while the abundance of *Bacteroidetes* was significantly higher than that of the control group, leading to a significant decrease in the F/B ratio. Furthermore, the abundance of *Firmicutes* and *Bacteroidetes* in the gut was related to the dietary fiber content. A study on dietary fiber intake and fecal microbiota showed that individuals with low dietary fiber intake had a gut abundance of *Firmicutes* that was twice that of individuals with high dietary fiber intake. In contrast, *Bacteroidetes* was significantly enriched in the high dietary fiber intake group,<sup>66</sup> and animal experiments had also indicated that dietary fiber improved energy homeostasis by regulating gut microbiota abundance and lowering the F/B ratio in obese mice.<sup>67</sup> Furthermore, a study had shown that n-3 PUFA in flaxseed could reduce the F/B ratio.<sup>68</sup> Flaxseed had a high content of dietary fiber and n-3 PUFA, so it had a better effect on the improvement of gut microbiota. At the genus level, our research found that compared to the control group, the intervention with flaxseed powder significantly improved the abundance of *Clostridium sensu stricto 1*, *Parasutterella*, *Eubacterium xylanophilum* group, *Lachnospiraceae NK4A136* group, and *Bifidobacterium* in the gut microbiota of the intervention group subjects. The abundance of *Clostridium sensu stricto 1* in the gut microbiota of NAFLD mice was significantly reduced and was negatively correlated with body weight, serum ALT and TG concentrations, insulin resistance, and liver weight.<sup>69</sup> *Parasutterella* had been shown to participate in bile acid metabolism by altering microbial metabolites, including bilirubin and bile acid derivatives, thus playing a role in maintaining bile acid homeostasis.<sup>70</sup> *Lachnospiraceae\_NK4A136\_group* is a type of probiotic associated with obesity and can produce butyrate and improve intestinal barrier function.<sup>71</sup> *Bifidobacterium* belongs to the *Actinobacteria* phylum and has the function of improving intestinal barrier function and preventing pathogen invasion, and is negatively correlated with the visceral fat content and degree of obesity.<sup>72</sup> It had also been demonstrated that *Bifidobacterium* abundance in the gut microbiota of NAFLD patients was inversely related to serum TC levels.<sup>73</sup> *Bifidobacterium* was also sensitive to a high-fiber diet, and the supplementation of dietary fiber led to its significant enrichment.<sup>74</sup> A multi-ethnic cross-sectional study found that the abundance of *Eubacterium xylanophilum* group

was negatively correlated with the total body fat content.<sup>75</sup> Animal studies had also shown that *Eubacterium xylanophilum* group contributed to the improvement of obesity by regulating the concentration of branched-chain amino acids in circulation.<sup>76</sup> Additionally, our results showed that flaxseed powder intervention significantly reduced the abundance of *Coriobacteriaceae\_UCG-00*. It has been reported that *Coriobacteriaceae\_UCG-002* can produce cytotoxic metabolites such as toluene and phenol, which impair intestinal barrier function.<sup>77</sup> Meanwhile, animal experiments showed that the abundance of *Coriobacteriaceae\_UCG-002* in the gut was significantly and positively correlated with body weight, blood glucose level, serum LDL-C level, serum leptin level, and hepatic TG level in NAFLD mice.<sup>78</sup> At the same time, the Spearman correlation analysis indicated that the differences in gut microbiota mentioned above were significantly correlated with numerous indices of glucose-lipid metabolism and liver function, suggesting that the improvement of flaxseed powder in patients with NAFLD was partially attributable to changes in the abundance of gut microbiota.

However, there were some limitations to this study. Firstly, in the current experiment, only one dosage of flaxseed powder for a 12-week intervention was used, so there is no awareness of the relationship between NAFLD improvement and the dosage and duration of flaxseed intervention. Previous animal experiments had shown that different doses of flaxseed powder might have different intervention effects, and higher doses of flaxseed powder and longer intervention time might produce better effects in patients with NAFLD.<sup>32,33</sup> Secondly, compared to liver biopsy, MRI-PDFF only provided information on changes in the liver fat content, but lacked information on inflammation, hepatocyte ballooning degeneration, and fibrosis. Multiple studies had shown that MRI-PDFF had a good correlation with histological grade changes in liver fat deposition, and MRI-PDFF could more comprehensively assess the fat content of the entire liver.<sup>79-81</sup> Thirdly, the included population was small, and future population studies with larger sample sizes might be needed to further confirm the effect of flaxseed powder on improving NAFLD patients. Finally, further animal experiments are needed in later studies to explore the specific mechanism through which flaxseed improves NAFLD.

## Conclusion

In conclusion, this randomized controlled clinical trial showed that flaxseed powder intervention for 12 weeks moderately reduced the liver fat content of NAFLD patients, while it also improved many indicators such as body composition, liver function, and lipid metabolism of NAFLD patients, and also increased the abundance of beneficial bacteria and decreased the abundance of harmful bacteria in the gut microbiota of NAFLD patients. This might be related to the rich content of ALA, n-3 PUFA, lignans, and dietary fiber in flaxseed. Dietary interventions had important potential in the prevention and treatment of NAFLD. Our study highlighted the potential of



flaxseed as an emerging superfood adjunct for the treatment of NAFLD.

## Abbreviations

ALA	$\alpha$ -Linolenic acid
ALT	Alanine aminotransferase
Apo A1	Apolipoprotein A1
Apo B	Apolipoprotein B
AST	Aspartate aminotransferase
BMI	Body mass index
CHE	Cholinesterase
DBIL	Direct bilirubin
FINS	Fasting insulin
F/B	<i>Firmicutes/Bacteroidetes</i>
GGT	Gamma-glutamyl transpeptidase
HDL-C	High-density lipoprotein cholesterol
IBIL	Indirect bilirubin
IHMS	International Human Microbiome Standard
LDL-C	Low-density lipoprotein cholesterol
LP(a)	Lipoprotein (a)
MRI-PDFP	Magnetic resonance imaging estimated proton density fat fraction
NAFL	Nonalcoholic fatty liver
NAFLD	Non-alcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
n-3 PUFA	n-3 polyunsaturated fatty acids
TBIL	Total bilirubin
TC	Total cholesterol
TG	Triglyceride

## Author contributions

LY, GS and LL: designed the research; YT, YZ, WL, QH, QZ and RZ: conducted the research; YT and YZ: analyzed the data; YT and LY: wrote the article; and YT, LY, GS and LL: had primary responsibility for the final content; and all authors read and approved the final manuscript.

## Data availability

The data described in the article, code book, and analytic code will be made available upon request.

## Conflicts of interest

The authors declare no conflict of interest.

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