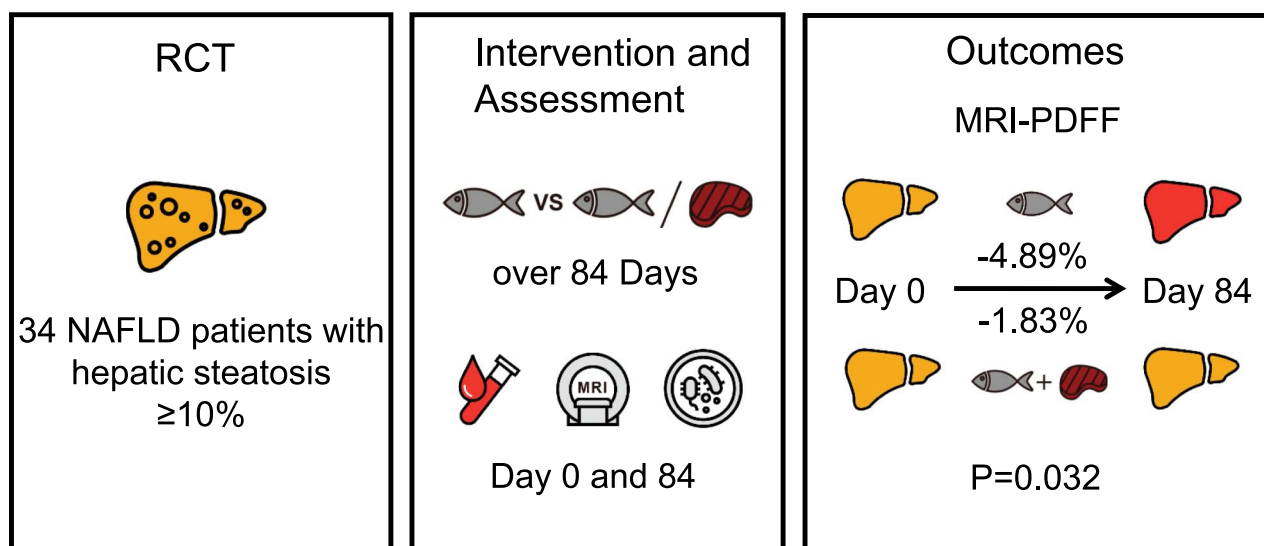LIVER

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A Freshwater Fish-Based Diet Alleviates Liver Steatosis by Modulating Gut Microbiota and Metabolites: A Clinical Randomized Controlled Trial in Chinese Participants With Nonalcoholic Fatty Liver Disease

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INTRODUCTION: **We aimed to assess the effects of 2 isoenergetic intervention diets (a freshwater fish-based diet [F group] or freshwater fish-based and red meat-based diets alternately [F/M group]) on liver steatosis and their relationship with intestinal flora in patients with nonalcoholic fatty liver disease (NAFLD).**

METHODS: **In this open-label, 84-day randomized controlled trial, 34 NAFLD patients with hepatic steatosis ≥10% were randomly assigned to the F group or F/M group in a 1:1 ratio using a computer-generated random number allocation by a researcher not involved in the study. Liver fat content and gut microbiota and its metabolites were measured.**

A freshwater fish-based diet alleviates liver steatosis by modulating gut microbiota and metabolites

RCT

34 NAFLD patients with hepatic steatosis ≥10%

Intervention and Assessment

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over 84 Days

Day 0 and 84

Outcomes

MRI-PDFF

Day 0 → Day 84

-4.89%

-1.83%

P=0.032

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RESULTS: At the end of intervention, the absolute reduction of hepatic steatosis was significantly greater in the F group than in the F/M group (-4.89% vs -1.83%, $P = 0.032$). Of the 16 secondary clinical outcomes, the improvement in 7 in the F group was greater compared with the F/M group, including alanine aminotransferase and gamma-glutamyl transferase. Furthermore, dietary freshwater fish and red meat consumption alternately did not exacerbate NAFLD. Moreover, changes in the enrichment of Faecalibacterium, short-chain fatty acids, and unconjugated bile acids and the depletion of Prevotella 9 and conjugated bile acids in the F group were significantly greater compared with the F/M group.

DISCUSSION: Higher intake of freshwater fish may be beneficial to NAFLD by regulating gut microbiota and its metabolites, whereas intake of a similar total of animal protein and fat from the alternating freshwater fish and red meat may not be harmful for NAFLD in the dietary management of patients with NAFLD.

INTRODUCTION

The prevalence of adult nonalcoholic fatty liver disease (NAFLD) has been estimated to be approximately 25% in the general population (1). The diagnosis and treatment of NAFLD is critical for the consequences that it can cause in the development of end-stage liver disease and hepatocellular carcinoma, as well as the correlated effect on the prevalence rate of metabolic syndrome, cardiovascular disease, and other malignant tumors (1,2).

No effective treatment is readily available on the NAFLD condition to date. On the other hand, lifestyle modifications, particularly a calorie-restricted diet, habitual physical activity, and weight loss, have been advocated for the treatment of NAFLD (3–5). However, sticking to a calorie-restricted diet and regular exercise can be challenging over the long term for most participants with NAFLD (6). Therefore, an alternative that is simple to implement and easier to adhere to is urgently needed. The ideal diet should effectively reduce liver steatosis, inflammation, and fibrosis, independent of weight loss, and can be maintained over the long term. Studies have shown that changes in dietary composition, such as increasing dietary fiber and omega-3 polyunsaturated fatty acid (n-3 PUFA) intake, can improve NAFLD, independent of dietary calorie restriction and body mass index (BMI) reduction (7).

Freshwater fish and red meat are the 2 main animal protein and fat sources among Chinese population. Epidemiological studies have shown that fish intake can reduce the occurrence of NAFLD, which may be related to the fact that fish is rich in n-3 PUFAs (8). However, the intake of red meat is positively related to the occurrence of NAFLD, potentially caused by the saturated fatty acids and heme iron present in the red meat (9,10).

In recent years, the gut microbiota has coevolved and has been interdependent with the host. Through the enterohepatic axis, gut microbiota plays an essential role in the occurrence and development of NAFLD (11). Moreover, it has been reported that different types of meat (fish and red meat) can potentially influence the gut microbial profile (12). Therefore, the effect of dietary fish and red meat on the occurrence of NAFLD can be potentially governed by the change in gut microbiota (9).

As mentioned above, NAFLD treatment is currently warranted and driven by comprehensive lifestyle intervention including a healthy diet. However, in the daily dietary management

of participants with NAFLD, priority is given to animal foods that can not only meet the daily animal protein and fat nutritional requirements of individuals but also be beneficial or be the least harmless for NAFLD. At present, no existing literature or research is available to address the abovementioned concerns. In this study, we proposed a novel randomized trial to facilitate the comparison of 2 isocaloric intervention diet choices (i.e., a freshwater fish-based diet or a freshwater fish-based and red meat-based diets alternately) on the metabolic phenotypes and to validate their relationship with intestinal flora in participants with NAFLD. We hypothesized that a freshwater fish-based diet would induce a greater improvement in hepatic steatosis by regulating gut microbiota and its metabolites compared with an alternating combination of freshwater fish-based and red meat-based diets.

METHODS

Study design and participants

This study was a randomized and open-label, controlled clinical trial, which was designed and conducted according to the Consolidated Standards of Reporting Trials guidelines (13). Participants eligible for this study were in the age range of 18 to 70 years, clinically diagnosed of NAFLD with a presence of hepatic steatosis ≥10% estimated by magnetic resonance imaging-proton density fat fraction (MRI-PDFF) as baseline. The exclusion criteria are listed in detail in Supplementary Digital Content 1 (see Supplementary Table 1, http://links.lww.com/AJG/C588).

Participants were recruited from gastroenterology and nutrition outpatients of the First Affiliated Hospital of Jinan University (a tertiary center in Guangzhou, China) from September 2019 to September 2020. Consents were obtained from all participants in a written format. The study protocol was approved by the institutional ethics review board of the First Affiliated Hospital of Jinan University (ethical approval department no. 031). The trial protocol was registered at www.chictr.org.cn/ with a registration number of ChiCTR1900025074. Participants who met all inclusion criteria were randomly assigned to either a freshwater fish-based diet (F group) or the combination of a freshwater fish-based diet and a red meat-based diet at a daily alternating frequency (F/M group) in a 1:1 ratio using a computer-generated random number allocation by a researcher not involved in the study. Because all participants were diagnosed with NAFLD at the

Table 1. Daily consumption of food groups at baseline and during intervention

Food groups (g)	F group		F/M group	
	Baseline (n = 17)	Intervention (n = 17)	Baseline (n = 17)	Intervention (n = 17)
Refined grains	266.18 ± 26.43[a,b]	269.41 ± 24.68[c]	254.71 ± 23.28[b]	258.24 ± 20.61
Red meat	90.00 (45.00, 120.00)[a]	0[d,e]	90.29 ± 33.93	143.24 ± 23.91[d]
Freshwater fish	89.71 ± 30.08[a]	344.12 ± 63.65[d,e]	87.65 ± 30.32	143.24 ± 16.48[d]
Poultry	100.00 (80.00, 110.00)[a]	0[d]	92.94 ± 27.33	0[d]
Eggs	24.47 ± 13.38[a]	0[d]	25.76 ± 11.65	0[d]
Dairy products	94.12 ± 86.61[a]	0[d]	84.71 ± 69.74	0[d]
Vegetables	377.76 ± 66.19[a,b]	380.12 ± 58.72[c]	359.41 ± 60.26[b]	353.53 ± 57.66
Fruits	265.29 ± 109.44[a,b]	274.59 ± 88.38[c]	255.29 ± 107.07[b]	251.76 ± 92.01
Vegetable oils	32.15 ± 2.69[a,b]	32.59 ± 3.34[c]	33.88 ± 4.26[b]	34.53 ± 3.91

Data are shown as mean ± SD or median (IQR).
F group, freshwater fish group; F/M group, freshwater fish/red meat group; IQR, interquartile range.
[a]$P > 0.05$ vs baseline of the F/M group.
[b]$P > 0.05$ vs intervention of the same group.
[c]$P > 0.05$ vs intervention of the F/M group.
[d]$P < 0.01$ vs baseline of the same group.
[e]$P < 0.01$ vs intervention of the F/M group.

outpatient clinic, they had previously received diet and exercise education about the daily management of NAFLD.

Dietary intervention

A detailed dietary plan for the enrolled participants during the intervention period was formulated with the assistance of a registered dietitian and tailored to the baseline diet (including total calories, macronutrient ratios, and habitual diet). The baseline diet was assessed by a 24-hour dietary recall method (2 weekdays and 1 weekend day). The calculation of the nutritional composition was based on the China Food Composition 2009 (14). In the F group, freshwater fish (i.e., alternation of bighead carp and grass carp on a daily basis) served as the only source of animal protein and fat in the usual diet. In the F/M group, a combination of freshwater fish (i.e., bighead and grass carp) and red meat (i.e., beef, pork, and mutton) with a daily alternation (namely, 1 cycle of this alternate diet is a 2-day period) served as the only source of animal protein and fat in the usual diet. The 2 intervention diets were intended to be isocaloric, and the overall intervention period was set for 84 days. The menu for the baseline diet and the intervention diets in the 2 groups is presented in Table 1. To ensure the consistency of nutrients, all freshwater fish and red meat (pork sirloin, beef sirloin, and mutton tenderloin) were purchased from the same market (Huifu West Road Market, Guangzhou, China) and delivered to the participants every 3 days during the intervention period.

Participants were given dietary guidelines on weighing, processing, cooking methods, and storage methods. In addition, participants were provided with kitchen scales and oil scales for maintaining consistency throughout the study (shown in Supplementary Digital Content 2 [see Supplementary Figure 1, http://links.lww.com/AJG/C589]). Participants were encouraged to contact the investigator by phone or message at any time for inquiry. To improve dietary adherence, researchers assessed participants' satisfaction with the diet through weekly phone calls and a total of 4 times home visits (see Supplementary Figure 2,

Supplementary Digital Content 2, http://links.lww.com/AJG/C589). In addition, the participants were allowed to dine out for 5 times during the intervention period (such as special holidays and family get-togethers). They were required to coordinate with the researchers and follow the recipe.

Clinical assessments

Study visits were conducted at baseline and on day 84 of the intervention. Each visit included medical history, anthropometric evaluation (including body mass index, body weight, and waist circumference), and fasting blood collection. Fecal samples were collected at baseline and on day 84 of the intervention. Participants were asked not to make any changes to their daily physical activity and to maintain body weight. On day 0, day 7, day 42, and day 84, the 7-day recall method was used to measure physical activity energy expenditure through an interview-administered survey instrument modified from the Cross-Cultural Activity Participation Study (15). Metabolic equivalent tasks (METs) were conducted to measure the physical activity energy expenditure of the participants (16). Further details about the physical activity assessment are present online (see Supplementary Methods, Supplementary Digital Content 3, http://links.lww.com/AJG/C590).

Primary clinical outcome

The primary clinical outcome was change in liver fat content as measured by MRI-PDFF in the F group compared with the change in the F/M group over 84 days. Recent studies have found that MRI-PDFF (Siemens 3.0T MAGNETOM Verio, Erlangen, Germany) can provide highly precise, accurate, noninvasive, reproducible, and quantitative estimation of liver fat content (17). It has been shown to correlate well with magnetic resonance spectroscopy ($r2 = 0.99$, $P < 0.001$) and steatosis grading in concurrent liver biopsies (18,19). All participants were required to fast for at least 4 hours before evaluation. The readers placed the region-of-interest co-localized the baseline and day 84 of the study. The measurement was performed by a trained radiologist

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Table 2. Dietary intake of nutrients at baseline and during intervention

Nutrients	F group Baseline (n = 17)	F group Intervention (n = 17)	F/M group Baseline (n = 17)	F/M group Intervention (n = 17)
Energy (kcal)	1874.59 ± 159.15[a,b]	1871.29 ± 173.14[c]	1873.05 ± 159.69[b]	1847.28 ± 158.78
Protein (g)	86.26 ± 12.85[a,b]	88.41 ± 11.67[c]	85.60 ± 12.21[b]	84.61 ± 12.64
Protein (%)	18.29 ± 1.80[a,b]	18.82 ± 1.70[c]	18.65 ± 1.17[b]	18.47 ± 1.28
Carbohydrate (g)	264.11 (259.75, 277.02)[a,b]	265.10 (251.87, 269.95)[c]	246.04 ± 25.42[b]	246.77 ± 23.95
Carbohydrate (%)	54.94 ± 2.36[a,b]	54.76 ± 3.17[c]	53.59 ± 2.03[b]	54.24 ± 2.39
Dietary fiber (g)	9.66 ± 2.70[a,b]	9.74 ± 2.42[c]	9.18 ± 2.20[b]	9.17 ± 1.94
Fat (g)	59.47 ± 5.27[a,b]	58.24 ± 5.98[c]	59.92 ± 7.11[b]	58.24 ± 6.11
Fat (%)	28.35 ± 1.46[a,b]	28.00 ± 1.87[c]	29.24 ± 2.05[b]	28.82 ± 1.81
SFAs (g)	16.30 ± 1.67[a]	12.57 ± 1.50[d,e]	16.46 ± 2.11	14.53 ± 1.91[d]
MUFAs (g)	23.19 ± 2.24[a]	25.18 ± 2.61[d,f]	23.42 ± 2.80	27.10 ± 2.41[d]
n-3 PUFAs (g)	0.82 ± 0.13[a]	1.55 ± 0.33[d,e]	0.81 ± 0.16	1.00 ± 0.15[d]
n-6 PUFAs (g)	8.09 ± 0.70[a]	9.69 ± 1.12[d]	8.09 ± 1.07	9.25 ± 0.92[d]
n-6/ n-3 PUFAs	10.10 ± 1.40[a]	6.46 ± 1.17[d,e]	10.23 ± 1.62	9.36 ± 1.03[d]
Cholesterol (mg)	400.34 ± 88.67[a]	305.01 ± 55.62[d,f]	430.89 ± 87.80	269.72 ± 81.08[d]
Calcium (mg)	487.11 ± 109.83[a,b]	470.52 ± 54.08[c]	462.42 ± 93.24	389.54 ± 46.56[d]
Magnesium (mg)	288.49 ± 32.51[a]	328.18 ± 44.21[d]	291.58 ± 40.49[b]	297.46 ± 30.12
Heme iron (mg)	2.27 ± 0.62[a]	1.39 ± 0.35[d,e]	2.36 ± 0.58	2.81 ± 0.50[d]
Non-heme iron (mg)	11.89 ± 1.41[a,b]	11.85 ± 1.29[c]	11.41 ± 1.34	12.59 ± 1.30[d]
Zinc (mg)	12.00 ± 1.53[a]	11.25 ± 1.01[d,e]	11.34 ± 1.39	13.34 ± 1.29[d]
Sodium (mg)	1,605.32 ± 136.76[a,b]	1,625.78 ± 146.60[c]	1,529.95 ± 123.69[b]	1,510.94 ± 104.38
Potassium (mg)	3,483.91 ± 189.94[a,b]	3,478.20 ± 182.61[c]	3,489.57 ± 262.01[b]	3,508.37 ± 240.46
Vitamin C (mg)	211.22 ± 70.57[a,b]	223.68 ± 89.91[c]	216.80 ± 48.82[b]	214.92 ± 40.47

Data are shown as mean ± SD or median (IQR).
F group, freshwater fish group; F/M group, freshwater fish/red meat group; IQR, interquartile range; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; SFAs, saturated fatty acids.
[a] $P > 0.05$ vs baseline of the F/M group.
[b] $P > 0.05$ vs intervention of the same group.
[c] $P > 0.05$ vs intervention of the F/M group.
[d] $P < 0.01$ vs baseline of the same group.
[e] $P < 0.01$ vs intervention of the F/M group.
[f] $P < 0.05$.

who was blinded to all clinical and biochemical data, including the grouping of participants. The scanning protocol and the sequence parameter settings about the MRI screening examinations are available online (see Supplementary Methods, Supplementary Digital Content 3, http://links.lww.com/AJG/C590).

Secondary clinical outcomes

The secondary clinical outcomes included the levels of body weight, waist circumference (WC), BMI, fasting blood glucose (FBG), fasting insulin (FINS), alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyl transpeptidase (GGT), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c), triglycerides (TG), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), C-reactive protein (CRP), and ferritin. All the results were compared within the same group for day 0 vs day 84, and the changes in them over the 84-day period between the 2 groups were evaluated. All these measurements were performed in the same laboratory.

The occurrence of adverse events was also monitored and is summarized (see Supplementary Methods, Supplementary Digital Content 3, http://links.lww.com/AJG/C590).

Gut microbiome, SCFA, and BA analysis

On day 0 and day 84, fecal samples were collected for bacterial 16S rRNA gene sequencing as well as short-chain fatty acid (SCFA) and BA quantitation. The collected stool samples were immediately stored in a −80 °C freezer until a freeze-drying procedure and analyzed. More detailed information are present online (see Supplementary Methods, Supplementary Digital Content 3, http://links.lww.com/AJG/C590).

Statistical analysis

As previously reported, a sample size of 16 participants per group (32 in total) would provide 90% power to detect 4% mean difference for liver fat fraction measured by MRI-PDFF, assuming an SD of 3% using a 2-sided test with a significance level of
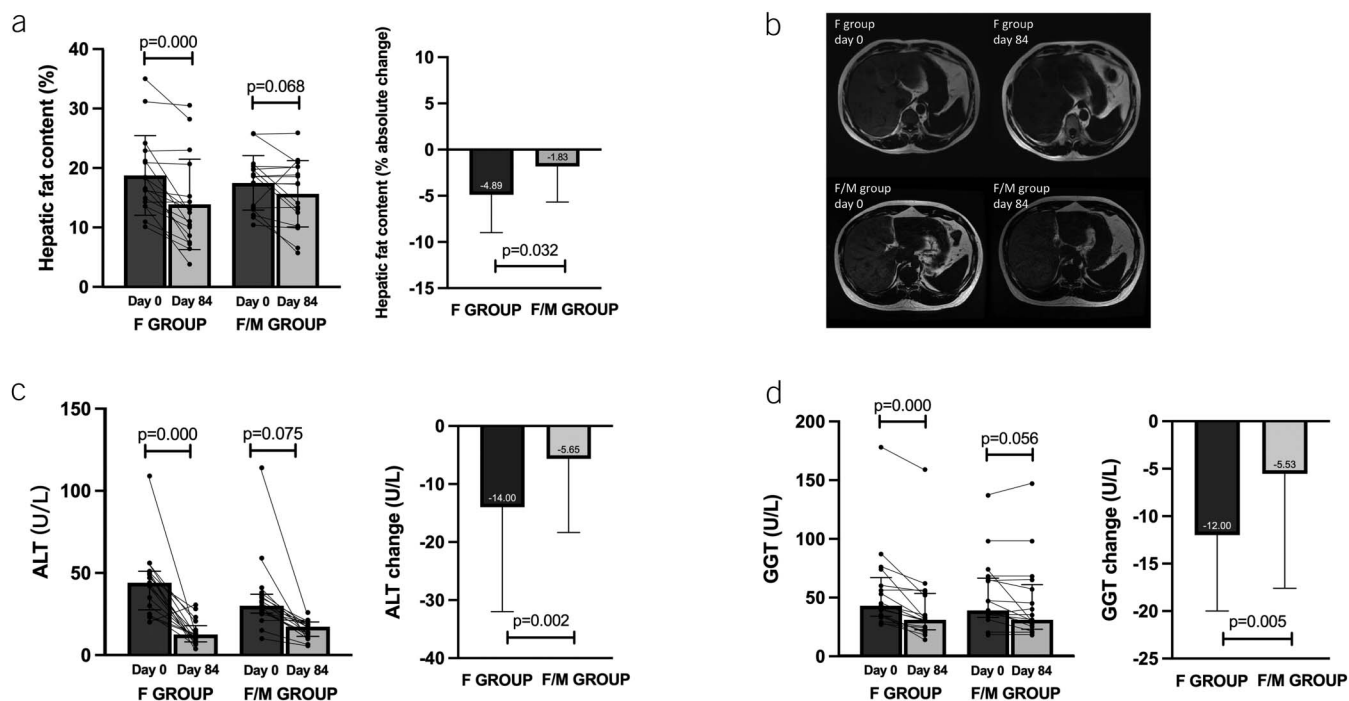
Figure 1. The freshwater fish-based diet induces significant improvements in hepatic fat content and liver enzyme. (**a**) Hepatic fat content measurement with MRI-PDFF. Boxes show means ± SDs. (**b**) MRI-PDFF images of hepatic fat content of a participants (male, 48 years old) in the F group that was decreased significantly from 23.9% at day 0 to 12.55% at day 84, and a participants (male, 31 years old) in the F/M group that showed no significant difference from 20.3% at day 0 to 20.2% at day 84. (**c**) Serum ALT level. Boxes show medians (IQRs). (**d**) Serum GGT level. Boxes show medians (IQRs). ALT, alanine aminotransferase; F group, freshwater fish group; F/M group, freshwater fish/red meat group; GGT, gamma-glutamyl transferase; PDFF, proton density fat fraction.

$P = 0.05$ and 20% drop-out rate (20). In addition, studies have shown that 4% changes in liver fat content are associated with histological changes in adult NAFLD (21).

The 1-sample Kolmogorov-Smirnov test was used to detect whether the continuous variables data and the differences between the 2 main time points (day 0 and day 84) were normally distributed. The relative changes of liver fat content were calculated as (the raw data of day 84 − the raw data of day 0)/the raw data of day 0 × 100%. The difference of other variables was calculated as the raw data of day 84 − the raw data of day 0. A paired Student t test or nonparametric 2-tailed Wilcoxon matched-pairs signed-rank test was selected to compare the 84th day and baseline values of each group according to the data distribution. Dichotomous variables data were evaluated using the χ^2 test. Outcomes were calculated on a strict intention-to-treat basis. Means and SDs and medians and interquartile ranges were used to represent normal and non-normal variable data, respectively. Classification variables were expressed as counts and percentages. All statistical analyses were performed using Statistical Package for Social Sciences (SPSS) software (version 27.0), and statistical significance was indicated by $P < 0.05$. Clustering correlation heatmap with sign was performed using the OmicStudio tools at https://www.omicstudio.cn. GraphPad Prism (version 9.0) was used for data visualization.

RESULTS

Baseline characteristics, diet, and physical activity of study participants

A total of 34 participants with NAFLD who met the inclusion criteria were enrolled in this study and randomly assigned to the F group or F/M group. During the intervention, 3 participants dropped out, resulting in a total of 31 participants who completed the diet intervention (see Supplementary Figure 3, Supplementary Digital Content 2, http://links.lww.com/AJG/C589). Three adverse events were reported during the study period but were unrelated to the intervention (see Supplementary Methods, Supplementary Digital Content 3, http://links.lww.com/AJG/C590). At baseline, demographic characteristics (age, sex, education, working condition, marital status, and family income) and metabolic phenotypes (liver fat content, liver enzymes, FBG, etc.) were not statistically different between the 2 groups (see Supplementary Tables 2–4, Supplementary Digital Content 1, http://links.lww.com/AJG/C588).

Compared with baseline, the F group had a significantly higher intake of freshwater fish without red meat, poultry, eggs, and dairy products while the F/M group had a significantly higher intake of red meat and freshwater fish without poultry, eggs, and dairy products during the intervention. Of note, a significantly higher intake of freshwater fish with no red meat was observed in the F group compared with the F/M group during the intervention (Table 1).

The nutritional composition of the baseline diets and the intervention diets consumed in the 2 groups is presented in Table 2. Compared with baseline, monounsaturated fatty acids (MUFAs), n-3 polyunsaturated fatty acids (PUFAs), n-6 PUFAs, and magnesium were significantly higher and saturated fatty acids (SFAs), n-6/n-3 PUFAs ratio, cholesterol, heme iron, and zinc were significantly lower in the F group, whereas MUFAs, n-3 PUFAs, n-6 PUFAs, heme iron, non-heme iron, and zinc were significantly higher, and SFAs, n-6/n-3 PUFAs, cholesterol, and calcium were

Table 3. Comparison of metabolic parameters at day 0 and day 84

Outcomes	F group (n = 17)			F/M group (n = 17)			P value
	Day 0	Day 84	P value	Day 0	Day 84	P value	
Hepatic steatosis assessed by MRI-PDFF, %	18.76 ± 6.70	13.87 ± 7.60	0.000	17.49 ± 4.58	15.66 ± 5.59	0.068	0.032[a] 0.041[b]
ALT, U/L	44.00 (27.50, 51.00)	21.00 (16.00, 35.00)	0.000	30.00 (25.50, 37.00)	28.00 (18.50, 35.00)	0.075	0.002[c]
GGT, U/L	43.00 (34.00, 67.00)	31.00 (22.50, 53.50)	0.000	39.00 (33.00, 66.50)	31.00 (23.00, 61.00)	0.056	0.005[c]
TG, mmol/L	2.63 ± 0.63	1.76 ± 0.58	0.000	2.32 ± 1.11	2.03 ± 1.01	0.147	0.014[c]
HDL-c, mmol/L	1.19 ± 0.26	1.27 ± 0.27	0.005	1.13 ± 0.13	1.10 ± 0.16	0.238	0.004[c]
FBG, mmol/L	5.57 (5.11, 5.81)	5.02 (4.55, 5.61)	0.001	5.34 (5.11, 6.19)	5.54 (5.10, 6.42)	0.397	0.002[c]
IL-6, pg/mL	2.77 ± 0.62	2.20 ± 0.38	0.001	2.73 ± 0.82	2.99 ± 1.09	0.129	0.001[c]
Ferritin, ng/mL	161.88 ± 105.43	148.86 ± 94.48	0.011	184.14 ± 134.39	189.80 ± 138.60	0.338	0.016[c]
Body weight, kg	72.50 (67.65, 87.05)	72.00 (66.95, 87.30)	0.123	79.50 (74.00, 92.50)	79.50 (73.00, 92.75)	0.086	0.683[c]
WC, cm	97.18 ± 9.95	93.59 ± 9.31	0.001	99.18 ± 10.77	96.47 ± 10.93	0.022	0.530[c]
BMI, kg/m^2	28.72 ± 3.82	28.18 ± 3.70	0.093	28.76 ± 4.58	28.44 ± 4.60	0.063	0.551[c]

The data are shown as mean ± SD for normal variables or median (IQR) for non-normal variables. For normally distributed data, paired or unpaired Student *t* tests were used to analyze intragroup and intergroup differences. For non-normally distributed data, the Wilcoxon signed-rank test or Mann-Whitney *U* test was used to analyze intragroup and intergroup differences. All tests were performed 2-tailed.

ALT, alanine aminotransferase; BMI, body mass index; F group, freshwater fish group; F/M group, freshwater fish/red meat group; FBG, fasting blood glucose; GGT, gamma-glutamyl transferase; HDL-c, high-density lipoprotein cholesterol; IL-6, interleukin 6; MRI-PDFF, magnetic resonance imaging-estimated proton density fat fraction; TG, triglyceride; WC, waist circumference.

[a] *P* value comparing the absolute reduction of liver fat content over 84 days between the 2 groups.
[b] *P* value comparing the relative reduction of liver fat content over 84 days between the 2 groups.
[c] *P* value comparing the changes of metabolic phenotypes over 84 days between the 2 groups.

significantly lower in the F/M group during the intervention. Notably, the F group had a significantly higher intake of n-3 PUFAs and cholesterol and a significantly lower intake of SFAs, MUFAs, n-6/n-3 PUFAs, heme iron, non-heme iron, and zinc compared with the F/M group during the intervention. There were no significant differences in total energy, macronutrients (fat, protein, and carbohydrate) and their proportion, dietary fiber (Table 2), and physical activity level (see Supplementary Table 5, Supplementary Digital Content 1, http://links.lww.com/AJG/C588) in the intragroup and intergroup comparisons of the 2 groups before and after the intervention.

Dietary freshwater fish consumption alleviates liver steatosis in participants with NAFLD

A significant reduction in liver fat content was detected at day 84 compared with day 0 in the F group ($P < 0.01$). It is important to also mention that a reduction in liver fat content at day 84 was observed as compared with day 0 in the F/M group although there was no significant difference in the change ($P > 0.05$). At the end of the intervention, the absolute decrease in hepatic steatosis was greater in the F group than in the F/M group (−4.89% vs −1.83%, $P = 0.032$) (Figure 1a; Table 3). In addition, the relative reduction in liver fat content was also significantly greater in the F group (−28.08%) than in the F/M group (−10.57%, $P = 0.041$) over the 84-day period (see Supplementary Figure 4A, Supplementary Digital Content 2, http://links.lww.com/AJG/C589). Of note, 7 (41.18%) and 5 (29.41%) participants showed a relative change of liver fat content of ≥30% on day 84 reported in the F group and in the F/M group, respectively (see Supplementary Table 6, Supplementary Digital Content 1, http://links.lww.com/AJG/C588). In

addition, 5 participants (29.41%) in the F group and 2 participants (11.76%) in the F/M group showed MRI-PDFF less than 10% on the 84th day of intervention (see Supplementary Table 6, Supplementary Digital Content 1, http://links.lww.com/AJG/C588). These results demonstrate that dietary freshwater fish consumption induces a greater improvement in hepatic steatosis compared with the combination diet of alternating freshwater fish and red meat consumption that may not exacerbate hepatic steatosis at least in participants with NAFLD.

Dietary freshwater fish consumption ameliorates several metabolic phenotypes in participants with NAFLD

From day 0 to day 84, the levels of ALT, GGT, TG, HDL-c, FBG, homeostasis model assessment of insulin resistance (HOMA-IR), IL-6, ferritin, and WC improved significantly, whereas there were no significant changes in AST, TC, LDL-c, FINS, CRP, TNF-α, body weight, and BMI in the F group. From day 0 to day 84, the changes in the levels of ALT, AST, GGT, TG, TC, LDL-c, HDL-c, FBG, FINS, HOMA-IR, CRP, TNF-α, IL-6, body weight, ferritin, and BMI were not significantly affected except for a significant reduction observed in the level of WC in the F/M group (Figures 1–3; Table 3; see Supplementary Table 7, Supplementary Digital Content 1, http://links.lww.com/AJG/C588; see Supplementary Figure 4, Supplementary Digital Content 2, http://links.lww.com/AJG/C589). The improvement in the levels of ALT, GGT, TG, HDL-c, FBG, IL-6, and ferritin was significantly greater in the F group compared with the F/M group over the 84-day period, whereas there were no significant differences observed in the changes in AST, TC, LDL-c, FINS, HOMA-IR, CRP, TNF-α, body weight, WC, and BMI between both groups (Figures 1–3;
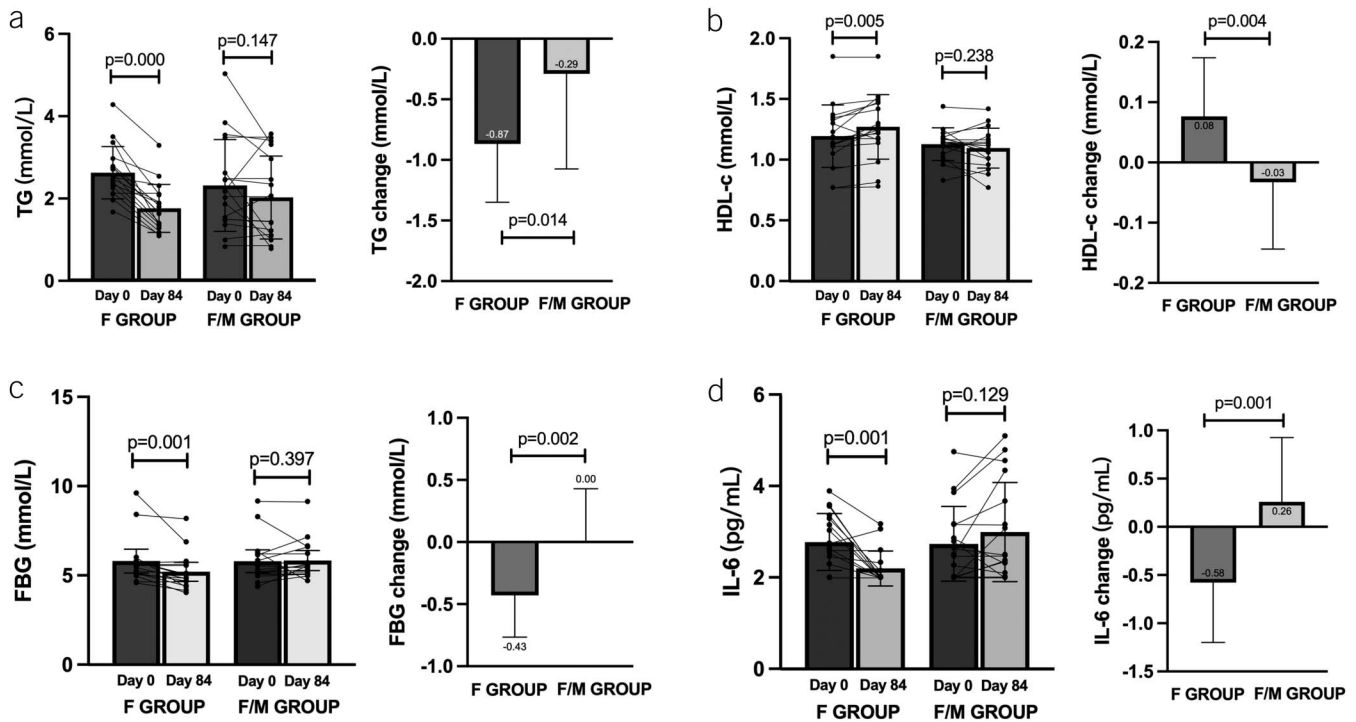
Figure 2. The freshwater fish-based diet significantly improves blood lipid, blood glucose, and inflammatory factors. (**a**) Serum TG level. Boxes show means ± SDs. (**b**) Serum HDL-c level. Boxes show means ± SDs. (**c**) Serum FBG level. Boxes show medians (IQRs). (**d**) Serum IL-6 level. Boxes show the means ± SDs. F group, freshwater fish group; F/M group, freshwater fish/red meat group; FBG, fasting blood glucose; HDL-c, high-density lipoprotein cholesterol; IL-6, interleukin 6; TG, triglyceride.

Table 3; see Supplementary Table 7, Supplementary Digital Content 1, http://links.lww.com/AJG/C588; see Supplementary Figure 4, Supplementary Digital Content 2, http://links.lww.com/AJG/C589). These results suggest that intake of freshwater fish significantly improves several clinical markers associated with NAFLD in addition to alleviating liver steatosis compared with the combination diet of alternating freshwater fish and red meat consumption in participants with NAFLD.

Dietary freshwater fish consumption partially redresses gut microbiota dysbiosis in the improvement of the metabolic phenotypes of participants with NAFLD

To explore the potential role of the gut microbiota in mediating dietary freshwater fish-induced improvement in hepatic fat content, 16S rRNA gene sequencing was performed in feces from the participants with NAFLD in both groups. At the phylum level, the dominating species in the gut microbiota (mean relative abundance >1%) were identified as Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Fusobacteria in both groups. From day 0 to day 84, the abundance of Proteobacteria was decreased significantly, from 13.16% to 5.62% ($P < 0.01$), whereas there were no significant differences in the abundances of Firmicutes, Bacteroidetes, Actinobacteria, and Fusobacteria in the F group. From day 0 to day 84, the abundances of Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Fusobacteria showed no significant change in the F/M group ($P > 0.05$). The change in Proteobacteria abundance in the F group was significantly greater than that in the F/M group ($P < 0.01$), whereas insignificant differences in the changes were detected in the abundances of Firmicutes,

Bacteroidetes, Actinobacteria, and Fusobacteria between the 2 groups over the 84-day period ($P > 0.05$). As for the abundance ratio of Firmicutes to Bacteroidetes, there was no significant difference between the 2 groups in the intragroup comparison and intergroup comparison (see Supplementary Table 8, Supplementary Digital Content 1, http://links.lww.com/AJG/C588; Supplementary Figure 5, Supplementary Digital Content 2, http://links.lww.com/AJG/C589).

At the genus level, the top 25 dominant genera are presented in Figure 2c. From day 0 to day 84, the abundance of Faecalibacterium was significantly increased, and the abundances of Prevotella 9 and Escherichia-Shigella were significantly reduced ($P < 0.01$). Yet there were no significant changes in the abundances of other genera ($P > 0.05$) in the F group. From day 0 to day 84, the abundances of the 24 genera listed above showed no significant change ($P < 0.05$), except for a significant decrease in the abundance of Escherichia-Shigella in the F/M group (see Supplementary Table 9, Supplementary Digital Content 1, http://links.lww.com/AJG/C588; Supplementary Figure 5, Supplementary Digital Content 2, http://links.lww.com/AJG/C589). The changes in the abundances of Faecalibacterium and Prevotella 9 in the F group were greater compared with the F/M group ($P < 0.01$), whereas no significant differences were observed in the changes in the abundances of other bacteria between the 2 groups over the 84-day period ($P > 0.05$) (see Supplementary Table 9, Supplementary Digital Content 1, http://links.lww.com/AJG/C588; Supplementary Figure 5, Supplementary Digital Content 2, http://links.lww.com/AJG/C589). Then, we conducted a correlation analysis to identify potential associations between bacterial abundance and metabolic phenotypes. We revealed that
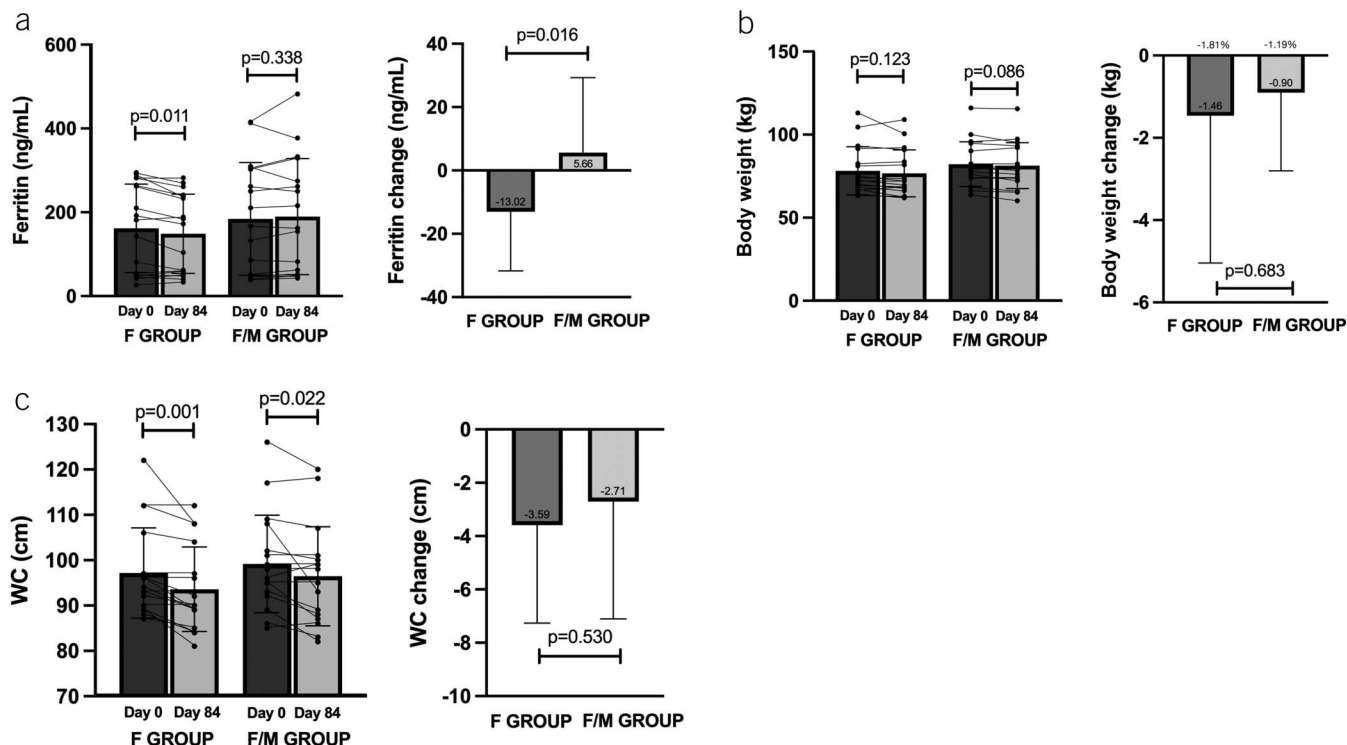
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Figure 3. The freshwater fish-based diet significantly improved ferritin levels and waist circumference. (**a**) Serum ferritin levels. Boxes show means ± SDs. (**b**) No significant change in body weight in both groups before and after intervention. Boxes show medians (IQRs). (**c**) Change of waist circumference. Boxes show means ± SDs. F group, freshwater fish group; F/M group, freshwater fish/red meat group; WC, waist circumference.

Faecalibacterium, which was enriched in fecal samples at day 84 in the F group, was negatively correlated with liver fat content and several metabolic parameters (ALT, TG, and IL-6). Escherichia-Shigella, Prevotella 9, and Megamonas were depleted in the participants at day 84 in the F group, which is related to the improvement of metabolic phenotypes (liver fat content, ALT, TG, TC, and HDL-c) (see Supplementary Figure 5, Supplementary Digital Content 2, http://links.lww.com/AJG/C589). These results suggest that gut microbiota changes induced by freshwater fish consumption may contribute to metabolic phenotypic improvement.

Dietary freshwater fish consumption improves NAFLD by inducing metabolites alternation

To reveal metabolites alternation, related to the gut microbiome, which is potentially involved in freshwater fish-induced NAFLD improvement, we performed metabolic profiling of feces from the participants. From day 0 to day 84, total SCFAs, propionic acid (PA), butyric acid (BA), and valeric acid (VA) quantities were significantly increased ($P < 0.01$), whereas acetic acid, isobutyric acid (IBA), isovaleric acid (IVA), and hexanoic acid were not significantly affected in the F group. From day 0 to day 84, AA was significantly increased ($P < 0.01$), whereas there were no significant changes in other SCFAs in the F/M group. The changes in total SCFAs, PA, BA, and VA in the F group were significantly greater than those in the F/M group, whereas there was no significant difference in the changes in AA, IBA, IVA, and hexanoic acid between the 2 groups over the 84-day period (see Supplementary Table 10, Supplementary Digital Content 1, http://links.lww.com/AJG/C588; see Supplementary Figure 6,

Supplementary Digital Content 2, http://links.lww.com/AJG/C589).

From day 0 to day 84, the levels of unconjugated BAs (chenodeoxycholic acid [CDCA], deoxycholic acid [DCA], and ursodeoxycholic acid [UDCA]) significantly increased and the levels of conjugated BAs (taurocholic acid [TCA], glycocholic acid [GCA], and glycochenodeoxycholic acid [GCDCA]) significantly decreased ($P < 0.01$) while insignificant differences were observed in other BAs in the F group. A decreasing trend of conjugated BAs (GCDCA) was observed from day 0 to day 84 while the levels of other BAs remained stable throughout the testing period in the F/M group. The changes of unconjugated BAs (CDCA, DCA, and UDCA) and conjugated BAs (TCA and GCA) in the F group were significantly greater than those in the F/M group, whereas there were no significant differences in the changes in other BAs over the 84-day intervention (see Supplementary Table 11, Supplementary Digital Content 1, http://links.lww.com/AJG/C588; see Supplementary Figure 7, Supplementary Digital Content 2, http://links.lww.com/AJG/C589).

Correlation analyses were performed to determine the potential associations between microbes and metabolites changes. Coincidentally, the results showed that Faecalibacterium enriched during the freshwater fish-based diet was positively correlated with the levels of SCFAs (BA and VA) and BA (cholic acid and DCA), but negatively correlated with BA (TCA, GCA, GCDCA, and Tβ-MCA) levels. Escherichia-Shigella depleted by the freshwater fish-based diet was negatively correlated with SCFA (VA) and BA (DCA and 12-KLCA) levels, but positively correlated with BA (taurochenodeoxycholic acid and Tβ-MCA) levels. Moreover, Prevotella 9 depleted by the freshwater fish-based diet was positively

correlated with the BA (TCA) level and negatively correlated with the SCFA (BA) level (see Supplementary Figures 6 and 7, Supplementary Digital Content 2, http://links.lww.com/AJG/C589). These results, taken together, suggest that the change in bacterial abundances modulated by the freshwater fish-based diet may be responsible for metabolites alternation, attributed to the improvement of metabolic phenotypes in participants with NAFLD.

DISCUSSION

In this dietary intervention study that lasted for 84 days, we demonstrated for the first time that intake of freshwater fish, which was the sole source of animal protein and fat in the usual diet, resulted in the greater improvements in liver fat content and other metabolic phenotypes (ALT, GGT, TG, HDL-c, FBG, IL-6, and ferritin) compared with the combinatory alternating freshwater fish and red meat consumption in participants with NAFLD without changes in total energy, macronutrients (fat, protein, and carbohydrate) and their proportion, dietary fiber, physical activity, and body weight. In addition, liver fat content and other metabolic phenotypes were not significantly affected, except for a significant reduction in WC at the end of the intervention in the F/M group. The abovementioned results suggest that a higher intake of freshwater fish in the usual diet may be beneficial for participants with NAFLD. By contrast, the intake of a similar total of animal protein and fat from the combination diet of alternating freshwater fish and red meat may not be harmful for NAFLD.

The compositions of the nutrient contents in the freshwater fish-based diet and the alternating freshwater fish-based and red meat-based diet might have caused the varying results on participants with NAFLD in this study. It has been shown that increased MUFA and n-3 PUFA in the diet have protective effects on NAFLD, obesity, and diabetes. A randomized controlled study showed that MUFA treatment could significantly reduce liver fat content and improve insulin sensitivity without changing body weight compared with the control group in participants with pre-diabetes (22). The meta-analysis by Yan et al. (23) revealed that n-3 PUFA can significantly improve liver fat, liver enzyme levels, insulin resistance, and blood sugar levels of participants with NAFLD. On the contrary, higher intake of SFAs, cholesterol, and heme iron has adverse effects on NAFLD and diabetes. Luukkonen et al. (24) reported that SFAs could stimulate fat de novo lipogenesis, worsening liver fat content and insulin resistance in overweight participants in a randomized controlled study. Animal studies have indicated that adding cholesterol to the diet can cause hepatitis and even inflammation of extrahepatic tissues while eliminating cholesterol in the diet improves non-alcoholic steatohepatitis in mice (25,26). Studies have shown that red meat is rich in heme iron, which is positively correlated with liver iron content and ferritin concentration (10). Liver iron content and ferritin concentration are associated with developing metabolic diseases, including NAFLD, insulin resistance, and type 2 diabetes (27,28). Furthermore, a higher n-6/n-3 PUFA ratio in the diet may increase the risk of chronic inflammatory diseases, such as NAFLD, obesity, and cardiovascular disease (29).

The aforementioned results were consistent with our findings that higher freshwater fish intake induced a significant improvement in liver fat content and related metabolic phenotypes in participants with NAFLD, which was associated with the elevated MUFA and n-3 PUFA as well as the reduced SFAs, n-6/n-3 PUFAs ratio, cholesterol, and heme iron in the freshwater fish-based diet. As for the F/M group, beneficial effects of increased MUFAs and n-3 PUFAs as well as decreased SFAs, n-6/n-3 PUFAs ratio, and cholesterol on participants with NAFLD possibly counterbalance the harmful effects of heme iron so that NAFLD-related metabolic phenotypes were not affected significantly after the 84-day dietary intervention. In addition, it is worth noting that the intervention diet in the F group contained more favorable dietary nutrients (increased n-3 PUFAs and decreased SFAs, n-6/n-3 PUFAs ratio, and heme iron and non-heme iron) except for 2 unfavorable nutritional components (increased cholesterol and decreased MUFAs) compared with the F/M group. This partly explains why the improvement of liver fat content and several metabolic indexes in the F group is greater than that in the F/M group at the end of the intervention.

There is a growing body of evidence that gut microbiota plays an important role in the occurrence and development of diabetes, obesity, cardiovascular disease, and NAFLD (30). Therefore, we explored the potential involvement of gut microbiota in mediating dietary freshwater fish-induced NAFLD improvement. In the F group, the abundance of Faecalibacterium increased while the abundance of Escherichia-Shigella and Prevotella 9 decreased at the end of the intervention. Of note, dietary freshwater fish consumption induced a significantly greater increase in Faecalibacterium and a significantly greater decrease in Prevotella 9 compared with the alternating combinatory freshwater fish and red meat consumption along with the improvement in liver steatosis and other NAFLD-related metabolic phenotypes. Faecalibacterium is one of the most abundant and important symbiotic bacteria in human gut microbiota. It produces butyric acid through fermentation of dietary fiber and plays an important role in maintaining intestinal health (31). Faecalibacterium was significantly reduced in participants with NAFLD and non-alcoholic steatohepatitis, associated with obesity or insulin resistance (32). Compared with mice fed with a high-fat diet alone, mice fed with a high-fat diet and transplanted with Faecalibacterium had significantly lower liver fat content and liver enzyme levels (33). In addition, several reports showed that Proteobacteria and Escherichia-Shigella were positively associated with the risk of NAFLD, inflammatory response, type 2 diabetes, and other diseases (34,35). Prevotella 9 belongs to Bacteroidetes, a group of the genus Prevotella. Several clinical studies have linked Prevotella to a range of diseases, including advanced liver fibrosis, liver cirrhosis, insulin resistance, type 2 diabetes, inflammation, and obesity (36–38). The aforementioned results support our observations in this study. Our results provide new insights into the potential roles of gut microbiota in dietary freshwater fish-induced NAFLD improvement.

In addition to gut microbiota, different metabolites produced by commensal bacteria could be involved in the pathogenesis and progression of NAFLD (39). Gut macrobiotic-related metabolites, including SCFAs, amino acid catabolites, and BAs, regulate hepatic steatosis and inflammation by signaling from their homologous receptors. We, therefore, attempted to clarify metabolites (i.e., SCFAs and BAs) that potentially mediate the beneficial impact of dietary freshwater fish consumption on NAFLD.

In recent years, many studies have demonstrated the role of SCFAs in the improvement of NAFLD, such as reducing proinflammatory cytokines, improving intestinal barrier function, and regulating immune function and glucose and lipid metabolism (40). Metabolomic analysis showed that on day 84 of the intervention, total SCFAs, PA, BA, and VA were enriched in the F

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group. Moreover, dietary freshwater fish consumption induced a significantly greater increase in total SCFAs, PA, BA, and VA compared with the combinatory diet of alternating freshwater fish and red meat consumption. Consistent with our findings, increased SCFAs were associated with the improvement of visceral fat content, blood lipids, and liver enzyme levels (41,42).

SCFAs not only provide energy for intestinal mucosal cells but also act as signal molecules to regulate human metabolism. First, SCFAs can affect the de novo synthesis of fat and improve liver fat content by binding with G protein-coupled receptor in the liver (43). Second, SCFAs can activate GPR43 and release glucagon-like peptide-2 to improve intestinal mucosal barrier function, inhibit inflammatory cytokines, and reduce the occurrence of chronic inflammation (44). Finally, SCFAs inhibit the progression of NAFLD by enhancing liver AMP-activated protein kinase phosphorylation, reducing fatty acid synthesis and oxidative stress, and improving insulin sensitivity (45). Collectively, our findings suggest that dietary freshwater fish consumption can promote SCFA production by beneficial intestinal bacteria, thereby leading to improvements in hepatic steatosis and other metabolic indicators.

The results of BA profiling indicate that the regulation of BA signal might be involved in the effect of dietary freshwater fish consumption on NAFLD. On day 84 of intervention, dietary freshwater fish consumption-mediated changes in the enrichment of unconjugated BAs (CDCA, DCA, and UDCA) and the depletion of conjugated BAs (TCA and GCA) were significantly greater compared with the combinatory diet of alternating freshwater fish and red meat consumption. Consistent with our findings, the increases in unconjugated BA and the decreases in conjugated BA were associated with the improvement of liver fat content, blood glucose, and lipid levels (46).

BA, as an important signal molecule, regulates the gene expression of glucose, lipid synthesis, and metabolism as well as energy expenditure by binding with farnesoid X receptor and G protein-coupled bile acid (BA) receptor 5 in the enterohepatic circulation (47). Therefore, the metabolism of BA is also believed to be closely related to metabolic diseases, such as dyslipidemia, NAFLD, type 2 diabetes, and obesity (48). Overall, our results suggest that consumption of freshwater fish modifies the BA spectrum, along with improvements in liver steatosis and other indicators.

Strengths and limitations

This study had several strengths. First, a randomized controlled design was used in this study. Second, the participants strictly followed the prescribed diet during the intervention period, and images of food were sent to the researchers at each meal to ensure the accuracy and implementation of the diet (see Supplementary Methods, Supplementary Digital Content 3, http://links.lww.com/AJG/C590). Third, hepatic fat content was assessed by MRI-PDFF, which is a precise, noninvasive, and nonradiation measurement method.

However, limitations were also noticed. First, the sample size was relatively small, and the study duration was relatively short. Second, liver biopsies were not used to determine changes in liver inflammation and fibrosis. Third, this study could not be double-blinded, in which both participants and investigators were aware of the interventions. Fourth, the dietary freshwater fish intervention showed a significant improvement in hepatic steatosis, but it was not reduced to the normal range.

In conclusion, this 84-day randomized, controlled pilot study in participants with NAFLD showed that the freshwater fish-based diet induces a greater improvement in hepatic steatosis and other metabolic phenotypes by regulating gut microbiota and its metabolites compared with the alternating freshwater fish-based and red meat-based diet, independent of weight change. In addition, the alternating freshwater fish and red meat consumption may not exacerbate NAFLD, more appropriate to fit the daily eating habits and food diversity for long-term implementation. However, these results should be considered preliminary, and large-scale controlled studies are required to confirm these findings.

Study Highlights

WHAT IS KNOWN

✓ Lifestyle interventions are preferred for NAFLD.

✓ Fish reduces NAFLD risk.

✓ Red meat increases NAFLD risk.

WHAT IS NEW HERE

✓ Freshwater fish improves NAFLD.

✓ Combined freshwater fish and red meat does not aggravate NAFLD.

✓ These may be altered by the gut microbiota.

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