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RESEARCH ARTICLE

Combination of dietary fiber and exercise training improves fat loss in mice but does not ameliorate MASLD more than exercise alone

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Abstract

Lifestyle interventions, such as diet and exercise, are currently the main therapies against metabolic dysfunction-associated steatotic liver disease (MASLD). However, not much is known about the combined impact of fiber and exercise on the modulation of gut-liver axis and MASLD amelioration. Here, we studied the impact of the combination of exercise training and a fiber-rich diet on the amelioration of MASLD. Male APOE*3-Leiden.CETP mice were fed a high-fat high-cholesterol diet with or without the addition of fiber (10% inulin) and exercise trained on a treadmill, or remained sedentary. Exercise training and fiber supplementation reduced fat mass gain and lowered plasma glucose levels. Only the combination treatment, however, induced fat loss and decreased plasma triglyceride and cholesterol levels compared with sedentary control mice. Exercise training with and without the addition of fiber had a similar ameliorating effect on the MASLD score. Only exercise without fiber decreased the hepatic expression of inflammatory markers. Fiber diet was mainly responsible for remodeling the gut microbial composition, with an increase in the relative abundance of the short-chain fatty acid (SCFA)-producing genera *Anaerostipes* and *Muribaculaceae*, whereas, surprisingly, exercise training alone and with fiber resulted in the highest increase of SCFA production. Overall, the combination of exercise training and dietary fiber decreases fat mass and improves glucose and lipid homeostasis but does not have an additional synergistic positive effect on liver health compared with exercise training alone.

NEW & NOTEWORTHY The combination of dietary fiber intake and exercise training has a synergetic beneficial effect on the metabolic health, resulting in fat loss, lowered blood glucose, and lowered plasma lipid levels in mice with steatotic liver disease. However, fiber supplementation, despite a positive remodulation of the gut-liver axis, does not have an additional positive effect on liver health compared with exercise training alone.

dietary fiber; exercise; gut microbiota; MASLD; mouse model

INTRODUCTION

Metabolic dysfunction-associated steatotic liver disease [MASLD, formerly known as NAFLD, nonalcoholic fatty liver disease (1)] is a growing global health burden, affecting more than 30% of the population (2). MASLD is characterized by lipid droplet accumulation in the liver and inflammatory processes, further leading to metabolic dysfunction-associated steatohepatitis (MASH) and liver cirrhosis (3). MASLD is a systemic disease, affecting whole-body metabolism, including intestinal function (4). MASLD induces gut dysbiosis, remodeling the gut microbial community. This may potentially lead to worsened disease progression via increased ethanol production (5) that further damages the liver, causing higher gut permeability (4) that allows more lipopolysaccharide (LPS) to enter the circulation (6) and higher inflammation levels in the gut (7). Although MASLD is reversible, especially when treated early, very few

pharmaceutical options are available (8). As a result, lifestyle interventions, such as diet and exercise training, remain the main treatment strategies. Optimization of such strategies with the aim of resolving gut dysbiosis and beneficially modulating microbiota composition is one key to improved disease treatment and outcomes (9).

Although increased physical activity and exercise training do not always lead to weight loss in patients with MASLD, it has been shown to improve liver health markers and inflammation independently of weight loss (10, 11). Exercise diversifies the gut microbiota composition, which further leads to its stabilization and dysbiosis resolution, partially by restoring the abundance of short-chain fatty acid (SCFA)-producing microbes (12). In line, we demonstrated that exercise training of mice, specifically late in the active period, increased the relative abundance of *Akkermansia*, *Lachnospiraceae*, and *Ruminococcaceae*, SCFA-producing genera (13, 14).



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Dietary supplementation with indigestible fiber is another lifestyle intervention that is gaining traction as a potential MASLD therapy (15, 16). Fiber by itself increases satiety (17), improves bowel movement, and increases gut passage time (18). More importantly, fiber remodels the gut microbiota composition (19) and serves as a precursor for the production of SCFAs (such as acetate, butyrate, and propionate) through microbial fermentation, which is associated with improved cardiometabolic outcomes (20, 21). SCFAs are associated with greater fat loss (22), improved insulin sensitivity (23), improved gut barrier function (24, 25), and decreased inflammation (25). Butyrate specifically leads to reduced appetite and increased brown adipose tissue activation (26), which is associated with favorable health outcomes (27).

However, little is known about the combination of fiber and exercise training as MASLD therapy and whether this could give a synergistic effect on disease amelioration. One study showed that a combination of fiber and exercise training caused fat loss in patients with obesity (28), which may be beneficial for MASLD, as 10% loss of body weight leads to a significant reduction in liver steatosis (29). As SCFAs increase skeletal muscle oxidative capacity during exercise, fiber supplementation could potentially increase the amount of consumed energy during the exercise (30). In addition, as the SCFA producers increase with exercise training, they would likely become more abundant with increased fiber intake, amplifying positive effects on liver health. Hence, here, we hypothesized that the combination of a single dietary fiber [inulin, a well-studied fiber that has consistently been shown to have beneficial cardiometabolic effects in mice (31–33)] and aerobic exercise (treadmill training) would provide a synergistic effect on the amelioration of MASLD and tested this in male APOE*3-Leiden.CETP mice, a well-established model for human-like lipid metabolism and cardiometabolic diseases including MASLD (14, 34, 35).

MATERIALS AND METHODS

Animal Experiments

Male APOE*3-Leiden.CETP mice were bred as previously described (36). The mice were littermates, raised in similar conditions before the start of the experiment. At the age of 8–12 wk, mice were group-housed (two to four mice per cage) under standard conditions (22°C, 12 h/12 h light/dark cycle) with ad libitum access to water and a high-fat high-cholesterol (HFHC) diet (60 kJ% fat + 1% wt/wt cholesterol; D12492, ssniff Spezialdiäten GmbH, Soest, Germany). After 6 wk of dietary acclimatization, based on body weight, body composition, and 4-h fasted plasma triglyceride (TG) and total cholesterol (TC) levels, mice were randomized into four treatment groups ($n = 18$ per group) using RandoMice version 1.0.9 (37). Two groups continued receiving the same HFHC diet (control groups), whereas the other two groups were switched to an HFHC diet with 10% inulin incorporated into the diet (fiber groups, in total fiber diet had 1% less of maltodextrin, 1% less of sucrose, 5% less of cellulose, and 7% more of inulin compared with HFHC diet; diet details can be found in Supplemental Data S1). Mice were kept on a 12 h/12h light/dark cycle, with ZT0 (lights on) at 03:30 AM for the control group and 04:45 AM for the fiber group. All mice had

standard cage enrichment, including nesting material, a paper roll toy, and a wooden chewing stick. Two groups exercise-trained on a treadmill for 8 wk at Zeitgeber time (ZT) 22 (RUN control and RUN fiber), whereas the other two groups remained sedentary (SED control and SED fiber) and were picked up, put on the researcher's hand, and returned to their home cages at the beginning of the running bout of their exercising counterparts to account for handling stress (Fig. 1A). At the end of the experiment, all mice were euthanized at ZT23, with half of the RUN mice being euthanized immediately after the last exercise bout, whereas the other half was euthanized 24 h after the last training bout. Mice were matched for fasting TG and TC, as well as body fat mass for the group division. All mice were euthanized using CO₂ before collection of portal vein blood, heart puncture blood, multiple organs [liver, subcutaneous white adipose tissue (sWAT), gonadal white adipose tissue (gWAT), caecum], and caecum content. Fifteen mice were excluded from further analysis due to the presence of liver tumors. The tumor distribution was independent of treatment and affected all groups similarly (three mice each from both control groups, five mice from Fiber SED, and four mice from Fiber RUN).

The animal experiment was carried out according to the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and was approved by the National Committee for Animal Experiments (Protocol No. AVD 11600202010187) and by the Ethics Committee on Animal Care and Experimentation of the Leiden University Medical Center (Protocol No. PE.21.002.037). METHODS were written in accordance with ARRIVE Guidelines 2.0 (38).

Sample Size Calculations

Required sample size was calculated with R version 4.1.1 assuming 25% difference between groups in MASLD score being considered statistically relevant, SD = 22%, $\alpha = 5\%$, and power = 80% for the two-way ANOVA with six multiple comparisons.

Humane Endpoints

During the training, mice were closely monitored for signs of injuries and removed from the treadmill after showing signs of fatigue (i.e., the lack of attempts to get back on the treadmill belt despite being nudged).

Blinding

The mouse experiment was not blinded due to the nature of the interventions. All further analysis with the mouse tissues was blinded.

Exercise Training Protocol

Mice were trained on a rat treadmill with five lanes (MazeEngineers, Skokie, IL), allowing two to four cage mates to run together per lane, as previously described (13), with minor adjustments. Briefly, after 3 days of treadmill acclimatization, mice were trained for 1 h per day, on five consecutive days per week for 8 wk. The training comprised a 15-min warm-up with speed increasing from 6 to 15 m/min, followed by 45-min exercise at 17 m/min. The exercise training took place under dim red light illumination during the dark phase at ZT22.

Body Weight, Body Composition, and Food Intake Assessment

Body weight of the mice and food intake per cage were assessed weekly at approximately ZT0. Every 4 wk, body fat and lean mass were measured by EchoMRI 100-Analyzer (EchoMRI, Houston, TX).

Plasma Lipid and Glucose Measurements

During week 8, mice were fasted for 4 h (ZT0 to ZT4). Subsequently, tail vein blood was collected into paraoxon-coated glass capillaries, and plasma was obtained after centrifugation for 5 min at 11,200 rcf and 4°C. Plasma TG and TC were measured using commercial enzymatic kits (10166588130 and 11489232216, respectively, Roche Diagnostics, Mannheim, Germany) as recommended by the manufacturer. Plasma glucose concentration was measured during the blood draw with a glucose meter (Accu-Chek, Roche, Basel, Switzerland). Insulin levels were measured using an Ultra-Sensitive Mouse Insulin ELISA kit (Crystal Chem, Zaandam, The Netherlands). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated with the following formula: [fasting glucose (mM) × fasting serum insulin (μU/mL)]/22.5 (39).

Liver and Adipose Tissue Histology

Liver tissue and sWAT tissue ($n = 14$ or 15) were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, and sectioned (5 μm). Liver and sWAT sections were stained with hematoxylin-eosin (H&E). MASLD score was determined in H&E-stained slides according to Liang et al. (40). Briefly, the scoring ranges from 0 to 8 and is evaluated semi-quantitatively through three criteria: microsteatosis, macrosteatosis, and hypertrophy. Each criterion is ranked from 0 to 3, where 0 = less than 5% of the tissue occupied, 1 = between 5% and 33% of the tissue occupied, 2 = between 33% and 66% of the tissue occupied, and 3 = greater than 66% of the tissue occupied. The maximum combination of macrosteatosis and microsteatosis results in a score of 5 (2 for one component and 3 for the other), resulting in a total maximal score of 8. For the staining of liver macrophages, endogenous peroxidase was blocked with H₂O₂, and the antigen was retrieved with proteinase-K (1.24568.0500, Merck, Rahway, NJ). Treated sections were then incubated with the primary antibody (Anti-F4/80 antibody ab6640, Abcam, Cambridge, UK) and a peroxidase-conjugated secondary antibody (MP-7444, Vector Laboratories, Newark) which was visualized using Vector NovaRED Substrate Kit (SK-4800). Nuclei were stained with Mayer's hemalum solution (1.09249, Sigma-Aldrich, St. Louis). Histological analyses were performed in eight randomly selected fields (~1.5 mm²) per sample for H&E staining determined by eye and in four randomly selected fields of the other stainings, which were calculated automatically. F4/80-positive area was determined with ImageJ software version 1.52a (see Supplemental Data S2 for the code). sWAT slides were stained with H&E, and the adipocytes' size was determined with ImageJ software (see Supplemental Data S2). Frozen cut liver sections were stained with Oil Red O staining (41), and the lipid-positive area was quantified with ImageJ software version 1.52a. For all of the stainings in the study, a mouse with the score

closest to the group's average is depicted in the representative screenshot.

Liver Lipid Quantification

Hepatic lipids were extracted from snap-frozen liver samples ($n = 14$ or 15 per group) using a modified protocol from Bligh and Dyer (34), and TG and TC were measured as aforementioned, and protein concentrations were measured using a commercial kit (Pierce BCA Protein Assay, Thermo Fisher Scientific, Waltham, MA). Hepatic lipid content was expressed as nmol per mg protein.

Gene Expression

Total RNA was isolated from snap-frozen tissues ($n = 14$ or 15 per group) using TriPure RNA Isolation Reagent (Roche Diagnostics, Mijdrecht, The Netherlands). cDNA was generated using Moloney Murine Leukemia Virus Reverse Transcriptase (Promega, Madison, WI). Quantitative real-time PCR was performed using GoTaq qPCR Master Mix (A6002, Promega, Madison, WI) with a Bio-Rad CFX384 Touch Real-Time PCR Detection System. mRNA expression was normalized to ribosomal protein lateral stalk subunit P0 (*Rplp0*) mRNA levels with the $\Delta\Delta C_t$ method (42) and expressed as fold change compared with the SED control. The primer sequences are listed in Table 1.

Plasma Short-Chain Fatty Acid Quantification

Five to six mice per group were used for the SCFA quantification. In RUN groups, only mice that were euthanized immediately after the last exercise bout were used. All mice of which the portal vein plasma was successfully collected were analyzed. In SED groups, six mice per group that had MASLD scores closest to the average were used. Acetic acid (C2), propionic acid (C3), isobutyric acid (C4), butyric acid (C4), 2-methyl-butyric acid (C5), isovaleric acid (C5), valeric acid (C5), and caproic acid (hexanoic acid, C6) were quantified by LC-MS/MS by Metabolon Inc. (North Carolina; Metabolon Method TAM148: "LC-MS/MS Method for the Quantitation of Short Chain Fatty Acid (C2 to C6) in Human Plasma and Serum"). Serum samples were spiked with stably labeled internal standards, homogenized, and subjected to protein precipitation with an organic solvent. After centrifugation, an aliquot of the supernatant was derivatized. An aliquot of the reaction mixture was injected onto an Agilent 1290/AB Sciex QTrap 5500 LC MS/MS system equipped with a C18 reversed-phase UHPLC column. The mass spectrometer was operated in negative mode using electrospray ionization (ESI). The peak area of the individual analyte product ions was measured against the peak area of the product ions of the corresponding internal standards. Quantitation was performed using a weighted linear least squares regression analysis generated from fortified calibration standards prepared concurrently with study samples. LC-MS/MS raw data were collected and processed using AB SCIEX software Analyst 1.7.3 and processed using SCIEX OS-MQ software v. 3.0.

Sequencing

Cecum content samples ($n = 14$ or 15 per group) were used for 16S sequencing. Qualified DNA (30 ng) isolated from

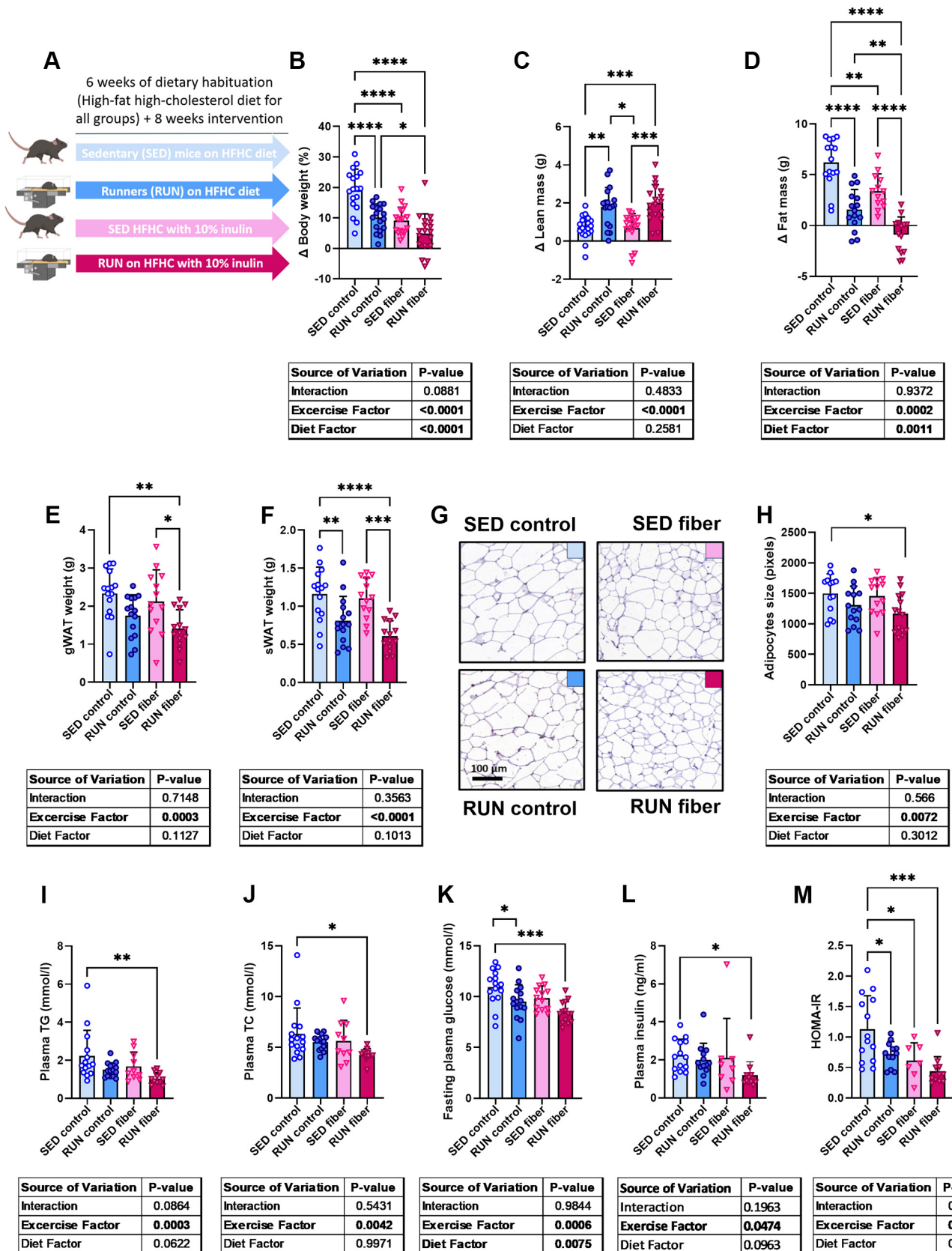


Table 1. The primers used for the quantitative PCR

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')
<i>Acs</i>	TGCCAGAGCTGATTGACATTC	GGCATACCAGAAGGTGGTGAG
<i>Adgre1</i>	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
<i>Col1a1</i>	GAGAGAGCATGACCGATGGATT	TGTAGGCTACGCTGTTCTTGCA
<i>Cyp7a1</i>	CAGGGAGATGCTCTGTGTTCA	AGGCATACATCCCTTCCGTGA
<i>Cyp8b1</i>	GGACAGCCTATCCTTGGTGA	CGGAACCTCCTGAACAGCTC
<i>Cyp27a1</i>	TCTGGCTACCTGCACCTTCCCT	CTGGATCTCTGGGCTCTTTG
<i>Fasn</i>	GCGCTCCTCGCTTGCTGCTCT	TAGAGCCCAGCCTTCCATCTCCTG
<i>Rplp0</i>	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
<i>Srebp1c</i>	AGCCGTGGTGAGAAGCGCAC	ACACCAGGTCCTTCAGTGATTGCT
<i>Tnfa</i>	GGCAGGTCTACTTTGGAGTCATTGC	ACATTCGAGGCTCCAGTGAATTCGG

Acs, acetyl-CoA synthetase; *Adgre1*, adhesion G protein-coupled receptor E1; *Col1a1*, collagen, type I $\alpha 1$; *Cyp7a1*, cytochrome P450 family 7 subfamily A member 1; *Cyp8b1*, cytochrome P450, family 8, subfamily B, polypeptide 1; *Cyp27a1*, cytochrome P450 family 27 subfamily A member 1; *Fasn*, fatty acid synthase; *Rplp0*, ribosomal protein lateral stalk subunit P0; *Srebp1c*, sterol regulatory element-binding protein 1; *Tnfa*, tumor necrosis factor α .

mouse cecum content and 16S v3-v4 pair-end Amplicon Sequencing primers (338 F forward primer: ACTCCTACGGG AGGCAGCAG; 806 R reverse primer: GGACTACHVGGGTW TCTAAT) were added for PCR. All PCR products were purified by Agencourt AMPure XP beads (Beckman Coulter, Inc., Brea, CA), dissolved in Elution Buffer, and eventually labeled to finish library construction. Library size and concentration were detected by Agilent 2100 Bioanalyzer. Qualified libraries were sequenced on the DNBSEQ-G400 platform according to their insert size. The QIIME 2 2021.8 tool was used to process the data and statistical analyses were performed in R version 4.1.1. Briefly, the raw sequence data were denoised and quality-filtered with the DADA2 plugin (via “denoise-paired”) (43). The obtained representative sequences were classified with the feature-classifier plugin (via “classify-sklearn”) (44) using the pre-trained Silva 138.1 animal-distal-gut taxonomy classifier. For statistical analyses, the Phyloseq package (version 1.36.0) (45) was used to integrate the counts matrix, taxonomy, and meta-data. To determine α -diversity (presented as Shannon index), the package’s function “estimate richness” was used and a one-way ANOVA was performed to compare the groups. Bray-Curtis dissimilarity index was used to calculate the β -diversity, with PERMANOVA testing to quantify the differences between the groups. For the identification of taxa changed between the groups, SIAMCAT (46) (version 1.12, abundance filtering = 0.01) was used with a false discovery rate correction = 0.05. Default parameters were used across analyses unless otherwise specified. Data were visualized with Ggplot2 (version 3.5.1).

Statistical Analysis

All individual data points are shown, and data are expressed as means $\pm$ SE, except for gene expression data, which is shown as geometric means $\pm$ geometric SD. Statistical analyses were performed using GraphPad Prism 9.01 (GraphPad, La Jolla, CA). Two-way ANOVA followed by Sidak’s multiple comparisons test was used, unless specified otherwise in the figure legends. The data were tested for normality. Statistical outliers

were removed after identification by Grubb’s test ($\alpha = 5\%$). Differences between groups were considered statistically significant if $P < 0.05$, $P < 0.01$, $P < 0.001$, or $P < 0.0001$.

RESULTS

Combination of Fiber Supplementation and Exercise Training Amplifies the Positive Effects of Separate Fiber or Exercise Treatment on Metabolic Parameters

To evaluate the impact of the combination of dietary fiber and exercise training on MASLD amelioration, mice were put on either control or fiber-enriched diet and either exercised (RUN) or remained sedentary (SED) for 8 wk (Fig. 1A). Both fiber and exercise training significantly decreased the body weight gain alone and in combination (Fig. 1B, Supplemental Fig. S1A). Both exercise training groups gained significantly more lean mass than the sedentary groups (Fig. 1C, Supplemental Fig. S1B), and both fiber and exercise training alone slowed fat mass gain compared with SED control, whereas the combination of fiber and exercise training led to fat mass loss (Fig. 1D, Supplemental Fig. S1C). No change in food intake was recorded throughout the study (Supplemental Fig. S1D). Only RUN fiber had a lower weight of gWAT compared with SED (Fig. 1E). Both exercise training groups decreased sWAT weight, with no changes between the sedentary groups with or without fiber supplementation (Fig. 1F). In accordance with fat loss, RUN fiber also had decreased adipocyte size in sWAT compared with SED control, whereas any differences between other groups were not significant (Fig. 1, G and H). In line, RUN fiber was the only group with decreased plasma TG and TC levels compared with SED control, though both exercise training groups had decreased fasting plasma glucose compared with SED control (Fig. 1, I–K). Finally, despite fasting insulin levels being significantly decreased only in RUN fiber (Fig. 1L), both fiber and exercise training alone and together decreased HOMA-IR compared with SED control (Fig. 1M). Overall, both fiber and exercise

Figure 1. The effect of dietary fiber supplementation, exercise training, and their combination on body composition and plasma parameters. A: after 6 wk of dietary habituation to a high-fat high-cholesterol diet, male MASLD-prone APOE*3-Leiden.CETP mice either exercised, received 10% dietary inulin supplementation, or a combination of both for 8 wk. The change in body weight (B), lean mass (C), and fat mass (D) was measured over these 8 wk. To better measure changes in fat mass, gWAT (E) and sWAT (F) weight was measured, and the size of sWAT adipocytes was assessed in H&E stained sections (G and H). Four hours fasted plasma TG (I), TC (J), and glucose (K) and insulin (L) levels were measured, and HOMA-IR calculated (M). Data are presented as means $\pm$ SE ($n = 14$ or 15); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ according to two-way ANOVA followed by Sidak’s multiple comparison test. gWAT, gonadal white adipose tissue; H&E, hematoxylin-eosin; HOMA-IR, homeostasis model assessment of insulin resistance; MASLD, metabolic dysfunction-associated steatotic liver disease; sWAT, subcutaneous white adipose tissue; TC, total cholesterol; TG, triglyceride.

training alone had positive effects on the general metabolic health, compared with SED control, whereas the combination of fiber and exercise training synergistically amplified these positive changes.

Exercise Training, Regardless of Fiber Supplementation, Ameliorates MASLD

To investigate the direct effect of the interventions on MASLD development, we studied liver-specific changes. Only RUN fiber had decreased liver weight compared with SED control, although exercise training was overall beneficial (Exercise factor $P = 0.0007$ in two-way ANOVA, Fig. 2A). Both exercise training groups had a similar reduction in MASLD score, whereas SED fiber did not differ from SED control (Fig. 2, B and C). Also for the reduction of the lipid-positive area as quantified with Oil Red O staining, and the hepatic TG content,

exercise training was overall beneficial (Exercise factor $P < 0.0001$ in two-way ANOVA), whereas fiber did not have any effect on the reduction of steatosis and steatosis-related parameters (Fig. 2, D–F). Curiously, despite exercise training alone or in combination with fiber having a similar effect on most of the liver measurements, only exercise training without fiber also decreased the hepatic TC levels compared with SED control, whereas RUN fiber did not (Fig. 2G). However, despite changes in TC concentration in the liver, there were no changes in the gene expression of the bile acid-synthesizing enzymes CYP7a1, CYP27a1, and CYP8b1 (Supplemental Fig. S1, E–H). Expression of *Hmgcr*, a gene encoding a protein involved in the endogenous cholesterol synthesis, was not changed either (Supplemental Fig. S1H). Finally, the expression of genes encoding enzymes in the de novo lipogenesis pathway, fatty acid synthase (*Fasn*) and sterol regulatory element-binding

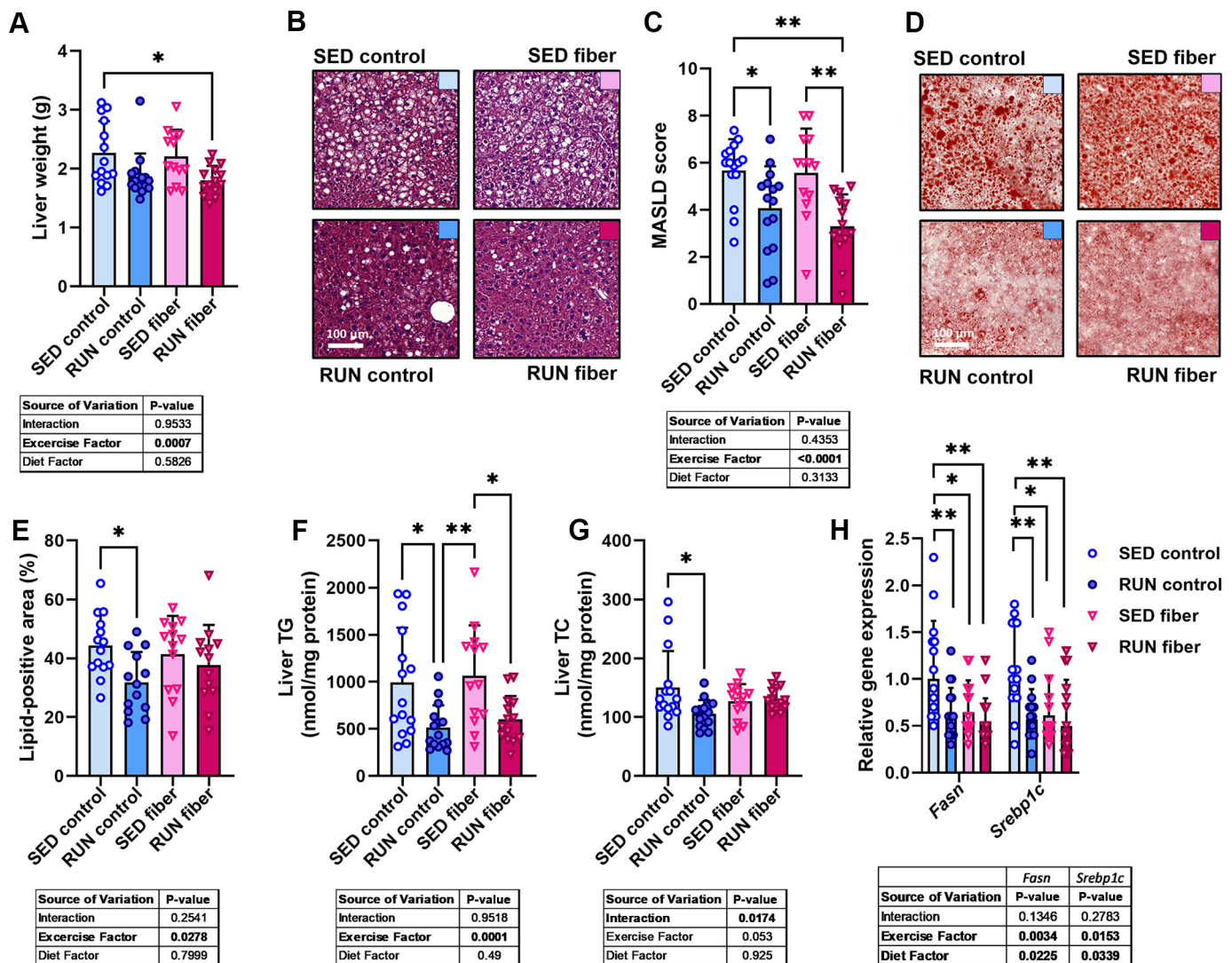


Figure 2. The effect of dietary fiber supplementation, exercise training, and their combination on MASLD development. To investigate the effect of the interventions on MASLD development, mouse livers were collected and weighed (A). H&E stained liver sections (B) were used to determine the MASLD score (C), and Oil Red O (ORO) stained sections (D) were used to quantify lipid-positive area in the liver (E). Subsequently, liver lipids were isolated, and liver TG (F) and TC (G) concentrations quantified. Finally, fatty acid synthase (*Fasn*) and sterol regulatory element-binding protein 1 (*Srebp1c*) expression in the liver was measured (H). Data are presented as means $\pm$ SE ($n = 14$ or 15) for A–G, and as geometric means $\pm$ geometric SD for H; * $P < 0.05$, ** $P < 0.01$, according to two-way ANOVA followed by Sidak's multiple comparison test. H&E, hematoxylin-eosin; MASLD, metabolic dysfunction-associated steatotic liver disease; RUN, runner; SED, sedentary; TC, total cholesterol; TG, triglyceride.

protein 1 (*Srebp1c*), was decreased similarly in all groups compared with SED control (Fig. 2H). Taken together, both exercise training with and without fiber supplementation decreased the MASLD score and hepatic lipid accumulation, whereas fiber alone did not have an effect on MASLD amelioration.

Only Exercise Training without Fiber Supplementation Decreases Liver Inflammation

As MASLD is an inflammatory disease, we next investigated inflammatory changes in the liver. Surprisingly, based on a staining, F4/80, a murine macrophage marker, tended to only be decreased in RUN control compared with SED control ($P = 0.052$), whereas the F4/80-positive area was comparable between the two fiber groups and SED control (Fig. 3, A and B). Similarly, the expression of adhesion G protein-coupled receptor E1 (*Adgre1*), the gene encoding F4/80 in mice, and of tumor necrosis factor α (*Tnfa*), encoding the pro-inflammatory cytokine TNF- α , were both decreased in RUN control compared with all other groups, whereas expression of collagen, type I $\alpha 1$ (*Col1a1*), a marker of fibrosis, was decreased only in RUN control compared with SED control (Fig. 3, C–E). The expression levels in both fiber groups remained unchanged compared with SED control (Fig. 3, C–E). These findings indicate that only exercise training without the addition of a high-fiber diet decreases liver inflammation under these experimental conditions.

Combination of Dietary Fiber and Exercise Training Changes Microbiome Composition and Enriches SCFA-Producing Bacteria

To gain insights into how the dietary intervention and exercise influence the gut microbiota composition in relation to disease outcomes, we conducted bacterial 16S sequencing of the cecum content of the mice. Fiber diet decreased the α -diversity, a metric of the diversity within

the sample (Fig. 4A). β -diversity, a metric of diversity between the samples, showed that the fiber diet was the main driver of microbial changes in the gut (Fig. 4B). After multiple testing adjustments, there was no difference between SED control and RUN control in microbiota composition, whereas the composition of RUN fiber was significantly different from all other groups, showing that the combination of fiber and exercise training had a bigger impact on the gut microbiota than fiber or exercise training alone (Fig. 4B). Changes on the phylum level support the finding that the fiber diet was the main cause of the observed changes, with both fiber groups increasing the abundance of *Actinobacteriota* and *Cyanobacteria*, while decreasing the abundance of *Proteobacteria* (Supplemental Fig. S2A). SED fiber also had a decreased abundance of *Firmicutes* (Supplemental Fig. S2A). On the family level, both fiber groups generally exhibited comparable changes. However, the *Lachnospiraceae* family was only increased in RUN fiber in comparison with all other groups (Supplemental Fig. S2B). The majority of this increase is explained by an enrichment of the *Anaerostipes* genus of the *Lachnospiraceae* family, which was absent in the control groups but constituted 20% of the total bacterial abundance in RUN fiber (Fig. 4C). *Muribaculaceae* was another notably increased genus in RUN fiber (Fig. 4C). Together, these SCFA producers accounted for more than 55% of the total relative abundance in the RUN fiber group (Fig. 4C). Their abundance was also increased to a lesser extent in SED fiber (Fig. 4C). On the contrary, the abundance of the *Alistipes* genus was decreased in RUN control compared with the SED control and was nearly completely depleted in both fiber groups (Fig. 4D). Other genera changes were in line with previous observations, with fiber being the main driver of changes on each level (Supplemental Fig. S3). Despite fiber being the main source for SCFA production, exercise training, and not fiber supplementation, was the main driver of the total SCFA increase in the portal vein after

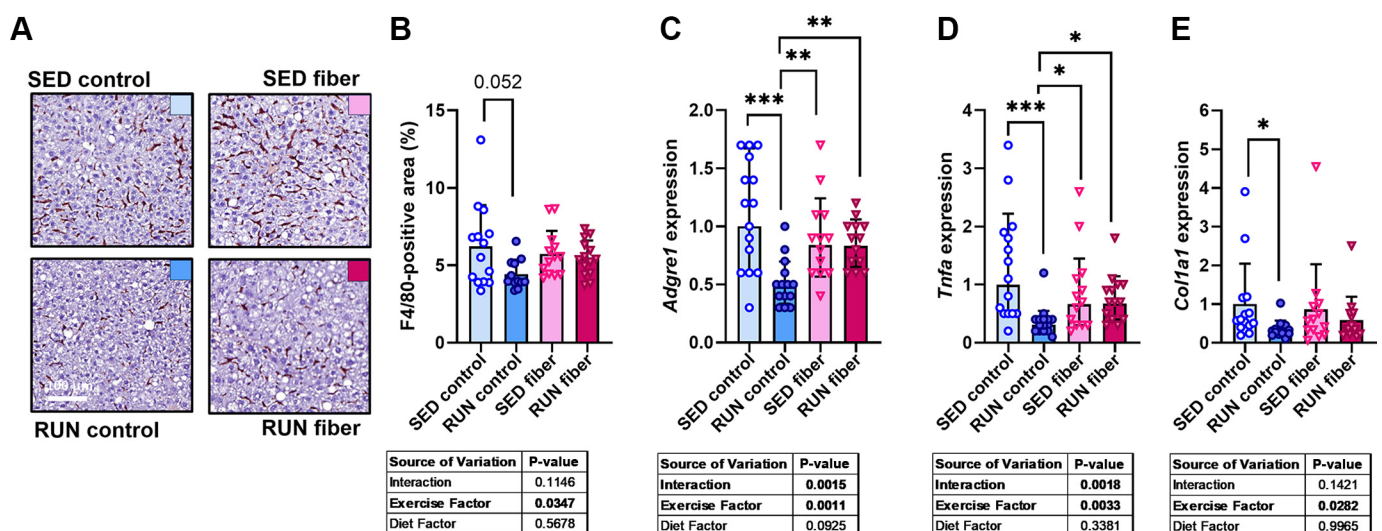


Figure 3. The effect of dietary fiber supplementation, exercise training, and their combination on liver inflammation in MASLD. F4/80 staining (A) was used to quantify the amount of liver macrophages (B). Subsequently, the expression of adhesion G protein-coupled receptor E1 (*Adgre1*), which encodes the murine macrophage marker F4/80 (C), tumor necrosis factor α (*Tnfa*), a liver inflammatory marker (D), and collagen, type I $\alpha 1$ (*Col1a1*), a marker of liver fibrosis (E) were measured. Data are presented as means $\pm$ SE ($n = 14$ or 15) for B and as geometric means $\pm$ geometric SD for C–E; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, according to two-way ANOVA followed by Sidak's multiple comparison test. MASLD, metabolic dysfunction-associated steatotic liver disease; RUN, runner; SED, sedentary.

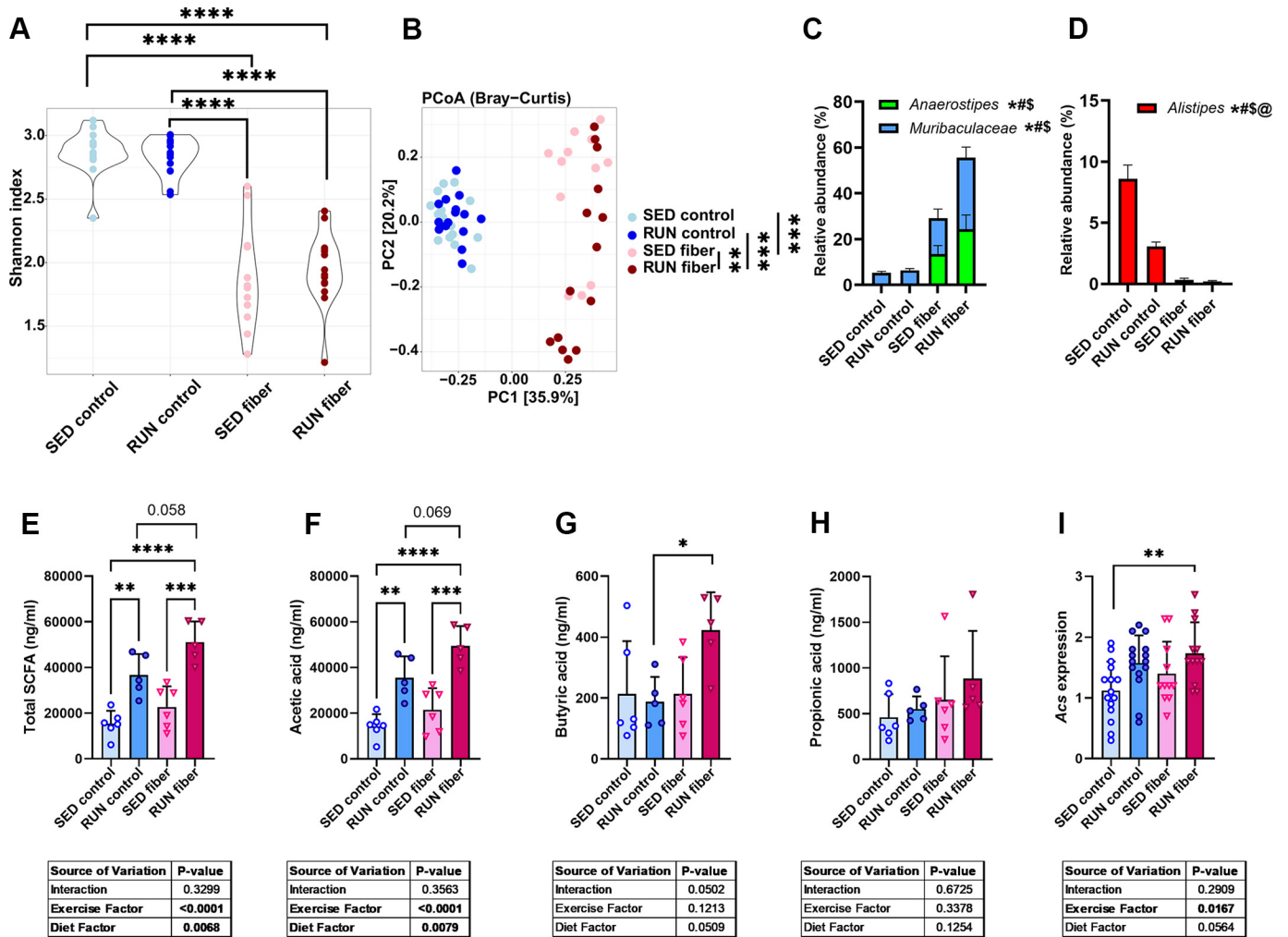


Figure 4. The effect of dietary fiber supplementation, exercise training, and their combination on gut microbiota composition and SCFA abundance. To investigate changes in the gut microbiota composition, we conducted 16S bacterial sequencing. α - (A) and β -diversity (B) were measured. Differences between the groups in A were calculated with one-way ANOVA with Tukey's post hoc correction, whereas differences in B were calculated with PERMANOVA with Bonferroni correction. The relative abundance of selected bacterial genera that were increased (C) or decreased (D) with the combination of fiber supplementation and exercise training was determined. Data is presented as an average value per group ($n = 14$ or 15), where: *significant difference between RUN fiber vs. SED control; #SED fiber vs. SED control; \$RUN fiber vs. RUN control; @RUN control vs. SED control, according to Wilcoxon test, adjusted for multiple testing. Subsequently, levels of total SCFAs (E), acetic acid (F), butyric acid (G), and propionic acid (H) were measured in the portal vein. Finally, the expression of acetyl-CoA synthetase (Acs) (I) in the liver was quantified. Data in E–H are presented as means $\pm$ SE ($n = 5$ or 6) and as geometric means $\pm$ geometric SD for I ($n = 14$ or 15); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, according to two-way ANOVA followed by Sidak's multiple comparison test. RUN, runner; SCFA, short-chain fatty acid; SED, sedentary.

exercise training (Fig. 4E). For instance, although SED control and SED fiber had total SCFA concentrations of only 15,000 and 23,000 ng/mL, respectively, RUN control had a concentration of 37,000 ng/mL, and RUN fiber had 51,000 ng/mL (Fig. 4E). The RUN fiber SCFA concentration constituted a more than twofold increase compared with SED fiber and also tended to be higher than RUN control ($P = 0.058$). This difference was mostly driven by acetic acid, which made up most of the total SCFA pool and showed the same trends (Fig. 4F). Curiously, butyric acid was only increased in the combination of fiber and exercise training group, whereas its levels in other groups remained similar to that of SED control (Fig. 4G). No detectable difference in propionic acid levels was observed (Fig. 4H). RUN fiber also showed a decreased concentration of 2-methylbutyric acid, whereas the levels

of hexanoic acid, isobutyric acid, isovaleric acid, and valeric acid were unchanged (Supplemental Fig. S4). The SCFA levels positively correlated with *Muribaculaceae* abundance (Supplemental Fig. S2, C–E). Finally, to investigate the impact of acetic acid on the liver, we measured the gene expression levels of acetyl-CoA synthetase (Acs), the enzyme incorporating acetic acid into the Krebs cycle, which tended to be increased in RUN control and was significantly increased in RUN fiber (Fig. 4I). This correlated with the highest acetic acid levels in the portal vein in RUN fiber (Supplemental Fig. S2F). Taken together, the combination of fiber and exercise training, which evoked the greatest improvement of whole-body metabolic homeostasis, led to the biggest change in the gut microbiome composition with a shift to a more SCFA-producing profile, which also resulted in higher blood levels of butyrate.

DISCUSSION

Dietary supplementation with fiber—in this study exclusively inulin—and exercise are both lifestyle interventions that are recommended for treating MASLD and other cardio-metabolic diseases. However, there is little evidence on whether combining them is more effective than either of them individually. Here, we show for the first time that although this combination is beneficial for fat loss and glucose and lipid homeostasis, it does not further improve MASLD amelioration compared with exercise training alone.

Noticeably, both interventions alone had a comparable effect on overall fat loss, adipocyte size, plasma triglycerides, cholesterol, and HOMA-IR, with exercise training being slightly more, albeit not significantly, effective than fiber supplementation. The combined effect on these parameters was, however, greater than that of the fiber or exercise training interventions alone for which there are a number of explanations. Combination treatment increased the concentrations of SCFAs in the blood, especially acetate and butyrate, the most. Their effect on fat loss may be mediated via an increase in energy expenditure (30, 47), as well as via an increase in muscle oxidative capacity (48). This would lead to more energy being used during exercise and would counteract hepatic lipid storage. In addition, butyrate by itself has been shown to have anti-obesity effects, and its increase only in the combination treatment group could have partially contributed to the observed fat loss effects (49–51). Finally, inulin by itself has been shown to be effective in reducing fasting glucose levels (52), which may be mediated by the suppression of resistin, a pro-inflammatory adipokine that modulates insulin function (53). Accordingly, the dietary fiber factor in this study significantly reduced blood glucose. Hence, it is conceivable that the combination of higher energy expenditure and fat loss was even more effective in improving glucose homeostasis.

However, despite these positive effects on whole-body metabolism, the combination treatment did not ameliorate MASLD progression or the amount of hepatic triglycerides more than exercise training alone. This is surprising, as this indicates that the liver does not store less lipids despite a smaller adipose tissue size. RUN fiber also had lower body weight and fat mass gain than RUN control, so it would be expected that by losing fat mass, MASLD would be ameliorated to a greater extent (29). One explanation for these similar steatosis levels is a pronounced increase in the production of acetate after the exercise training in the RUN fiber group, exceeding that of RUN control. Although generally SCFAs (specifically butyrate) are considered to be health-promoting, increased acetate levels in patients with MASLD were previously seen to correlate with a worsening disease prognosis (54, 55). Given that the majority of acetate entering the portal vein is immediately taken up by the liver, most of the acetate then enters the Krebs cycle. Correspondingly, the expression of *Acs*, a gene encoding the enzyme responsible for converting acetate into Acetyl-CoA, which is indispensable for de novo lipogenesis, was increased in RUN fiber (55) and correlated with acetate levels. This may explain the replenishment of lipid content in the liver after the exercise training, which results in a lack of clear differences between the two exercise training groups. Further studies

should investigate if high acetate concentrations derived from the gut lead to deteriorating liver health, and acetate labeled with stable isotopes can be used to investigate its metabolism in the liver in these high concentrations. However, clearly, exercise training overall is effective for MASLD amelioration.

Contrary to the hepatic TG decrease, only exercise training without fiber supplementation decreased inflammation in the liver, whereas the combination treatment had no effect. Cholesterol may play a role in the development of hepatic inflammation (56, 57), but in the current study, we did not see changes in the hepatic cholesterol content between exercise training and combined treatment groups. Unlike other insoluble fibers, water-soluble inulin has also been shown to exacerbate inflammation in the gut with a high-fat diet, which could have also led to an increased inflammation in the liver (58). Another cause for the unchanged liver inflammation in the combined treatment group can be the elevated amount of acetate which was shown to contribute to low-grade inflammation in the liver and other tissues (54). Taken together, inulin itself and elevated acetate levels may counteract the reduction of liver inflammation achieved with exercise training.

Curiously, although exercise training or the combination treatment ameliorated MASLD and fiber supplementation alone did not, the fiber diet was the determining factor in modulating the gut microbiota changes. First, inulin decreased the α -diversity, possibly due to it being the predominant source of energy for the bacteria and, hence, eliminating bacteria that cannot use it as a fuel. Second, fiber supplementation resulted in a β -diversity shift, with both fiber groups showing clear differences compared with the control groups. Although there was a significant difference between SED fiber and RUN fiber in β -diversity, there were very few significant differences between them on the genus level, indicating that exercise had a smaller effect on the microbiota changes. Curiously, two genera, *Anaerostipes* and *Muribaculaceae*, constituted nearly 55% of the total abundance in RUN fiber group. *Anaerostipes* genus is a known butyrate producer (59, 60), which can explain the increase in butyrate production in RUN fiber compared with other groups. As ~95% of butyrate is absorbed by colonocytes in the gut (49), butyrate could have had positive effects on the gut, which were not investigated in this study. *Muribaculaceae* is also known to negatively correlate with MASLD (61) and positively correlate with propionate (62) and acetate levels (63). Although we did not see an increase in propionate, *Muribaculaceae* abundance correlated with concentrations of total SCFAs, including propionate and acetate. Here, to specifically investigate which metabolites reach the liver, we only measured the changes in SCFA levels in the portal vein. However, as the described changes in SCFAs may also play a role in colonocyte health and in microbial community metabolism, future studies that measure SCFAs in the caecum are warranted.

Despite the diet remodeling the microbiota composition, exercise was the driving factor in the increase of total SCFAs. SCFA levels were measured in portal vein blood that was collected right after the exercise bout. Hence, one possible explanation is that the lactate produced by active skeletal

muscles entered the gut where it was readily converted into SCFAs (64–67), which led to an increase of SCFAs in the portal vein. It has been previously reported that lactate is converted to propionate or acetate in the gut of exercising athletes (65). In our study, the two most abundant bacterial genera, *Muribaculaceae* and *Anaerostipes*, have the capacity to convert lactate into acetate (63) and butyrate (68), respectively, which may explain the lack of increase in propionate. It is also possible that lactate-derived metabolites, including propionate, are further converted into other SCFAs by other members of the gut microbiota (69). Another question is whether the observed changes in SCFA levels are acute or long-lasting due to prolonged exercise training. We have previously shown that prolonged exercise training does not permanently increase SCFA concentrations in the portal vein (14), further suggesting that the acute SCFA increase is specifically exercise-mediated. This postexercise SCFAs increase would also explain why exercise training or the combination treatment improved MASLD and other metabolic parameters, whereas the fiber diet by itself had a much smaller effect, as the production of SCFAs was not significantly increased in SED fiber compared with SED control.

Conclusions

Overall, we show for the first time that although a combination of dietary fiber and exercise training is an effective strategy for weight loss, it does not synergistically contribute to MASLD amelioration compared with exercise training alone. Dietary fiber was the main factor in reshaping the gut microbiota composition, but exercise training, and not fiber supplementation, promoted an increase in SCFAs influx into the liver. The lack of the additional positive effects on the liver that are expected with an increase in fiber consumption may be explained by inulin being the only fiber tested here. Supplementing only one type of fiber decreased the bacterial diversity, possibly weakening the positive effects on the gut observed in other studies with mixed fiber supplementation. In addition, inulin itself may have some side-effects worsening MASLD development, such as increased inflammation in the gut or increased lipogenesis in the liver. Hence, future studies should include more diverse fiber mixtures and experiments in gnotobiotic models to verify the causality of the gut-liver axis impact on the disease development. However, with or without fiber supplementation, exercise training should be a crucial pillar in the treatment of MASLD, with its clear benefits for both the gut and the liver.

DATA AVAILABILITY

The 16S data can be accessed via the ZENODO database (<https://doi.org/10.5281/zenodo.13810371> and <https://doi.org/10.5281/zenodo.13810601>).

SUPPLEMENTAL MATERIAL

Supplemental Figs. S1–S4: <https://doi.org/10.6084/m9.figshare.28333988.v1>.

Supplemental Data S1: <https://doi.org/10.6084/m9.figshare.28333991.v1>.

Supplemental Data S2: <https://doi.org/10.6084/m9.figshare.28333985.v1>.

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DISCLAIMERS

During the preparation of this work, the authors did not use any generative AI tools for any parts of the submitted manuscript.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

A.K., P.C.N.R., and M.S. conceived and designed research; A.K., M.M.C., A.B., and M.S. performed experiments; A.K., M.M.C., A.B., and M.S. analyzed data; A.K., M.M.C., A.B., Q.R.D., P.C.N.R., and M.S. interpreted results of experiments; A.K., M.M.C., A.B., and Q.R.D. prepared figures; A.K. drafted manuscript; A.K., Q.R.D., P.C.N.R., and M.S. edited and revised manuscript; A.K., M.M.C., A.B., Q.R.D., P.C.N.R., and M.S. approved final version of manuscript.

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