Role of gut-liver axis in circadian exercise and dietary interventions to improve metabolic health

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Chapter 6

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:

Background: 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. **Methods:** 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. **Results:** 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 to 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*, while, surprisingly, exercise training alone and with fiber resulted

in the highest increase of SCFA production. **Conclusion:** 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 to exercise training alone.

New and 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 to exercise training alone.

Keywords: MASLD, inulin, dietary fiber, exercise, gut microbiota, mouse model

Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD, formerly known as NAFLD, non-alcoholic 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). While 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).

While 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).

Dietary supplementation with indigestible fiber is another lifestyle intervention that is gaining traction as 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). Additionally, as the SCFAs 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 prior to the start of the experiment. At the age of eight to twelve weeks, 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% w/w cholesterol; D12492, ssniff Spezialdiaten GmbH, Soest, Germany). After six weeks of dietary acclimatization, based on body weight, body composition and 4-hour 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), while the other two groups were switched to a 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 to HFHC diet; diet details can be found in appendix 2). Mice were kept on a 12h/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 eight weeks at *Zeitgeber* time (ZT) 22 (RUN control and RUN fiber), while 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 (Figure 1A). At the end of the experiment, all mice were sacrificed at ZT23, with half of the RUN mice being sacrificed immediately after the last exercise bout, while the other half was sacrificed 24 hours 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 sacrificed using CO_2 before collection of portal vein blood, heart puncture blood and multiple organs (liver, subcutaneous adipose tissue (sWAT), gonadal white adipose tissue (gWAT), caecum) and caecum content. 15 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 (3 mice each from both control groups, 5 mice from Fiber SED and 4 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). The methods section was 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 6 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

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, Illinois, USA), allowing two to four cage mates to run together per lane, as previously described (39), with minor adjustments. Briefly, after three days of treadmill acclimatization, mice were trained for 1 hour per day, on five consecutive days per week for eight weeks. The training consisted of a 15 minutes warm-up with speed increasing from 6 m/min to 15 m/min, followed by 45 minutes 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 four weeks, body fat and lean mass were measured by EchoMRI 100-Analyzer (EchoMRI, Houston, Texas, USA).

Plasma lipid and glucose measurements

During week eight, mice were fasted for 4 hours (ZT0 to ZT4). Subsequently, tail vein blood was collected into paraoxon-coated glass capillaries, and plasma was obtained after centrifugation for 5 min at 11200 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 (40).

Liver and adipose tissue histology

Liver tissue and sWAT tissue (n=14-15) 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.* (41). 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 = <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 = >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 H_2O_2, and the antigen was retrieved with proteinase-K (1.24568.0500, Merck, Rahway, New Jersey USA). 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, USA) which was visualized using Vector NovaRED® Substrate Kit (SK-4800). Nuclei were stained with Mayer's hemalum solution (1.09249, Sigma-Aldrich, St Louis, USA). Histological analyses were performed in eight randomly selected fields (~1.5 mm^2)

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 appendix 3 for the code). sWAT slides were stained with H&E, and the adipocytes size was determined with ImageJ software (see appendix 3). Frozen cut liver sections were stained with Oil Red O staining (42) and the lipid-positive area was quantified with ImageJ software version 1.52a. For all of the stainings in the manuscript, 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=14-15 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, Massachusetts, USA). Hepatic lipid content was expressed as nmol per mg protein.

Gene expression

Total RNA was isolated from snap-frozen tissues (n=14-15 per group) using TriPure RNA Isolation Reagent (Roche Diagnostics, Mijdrecht, The Netherlands). cDNA was generated using Moloney Murine Leukemia Virus Reverse Transcriptase (Promega, Madison, Wisconsin, USA). Quantitative real-time PCR was performed using GoTaq® qPCR Master Mix (A6002, Promega, Madison, Wisconsin, USA) 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 Ct method (43) and expressed as fold change compared with the SED control. The primer sequences are listed in Table 1.

Gene	**Forward primer (5'→3')**	**Reverse primer (5'→3')**
Acs	TGCCAGAGCTGATTGACATTC	GGCATACCAGAAGGTGGTGAG
Adgre1	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
Col1a1	GAGAGAGCATGACCGATGGATT	TGTAGGCTACGCTGTTCTTGCA
Fasn	GCGCTCCTCGCTTGTCGTCT	TAGAGCCCAGCCTTCCATCTCCTG

Rplp0	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
Srebp1c	AGCCGTGGTGAGAAGCGCAC	ACACCAGGTCCTTCAGTGATTTGCT
Tnfa	GGCAGGTCTACTTTGGAGTCATTGC	ACATTCGAGGCTCCAGTGAATTCGG

Table 1. The table shows the primers used for the quantitative PCR.

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 sacrificed immediately after the last exercise bout were used. All mice of which portal vein plasma was successfully collected were analyzed. In SED groups, 6 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, USA; 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 was collected and processed using AB SCIEX software Analyst 1.7.3 and processed using SCIEX OS-MQ software v3.0.

16S sequencing

Caecum content samples (n=14-15 per group) were used for 16S sequencing. 30 ng qualified DNA isolated from mouse caecum content and 16S v3-v4 pair-end

Amplicon Sequencing primers (338F forward primer: ACTCCTACGGGAGGCAGCAG; 806R reverse primer: GGACTACHVGGGTWTCTAAT) were added for PCR. All PCR products were purified by Agencourt AMPure XP beads (Beckman Coulter, Inc, Brea, California, USA), 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") (44). The obtained representative sequences were classified with the feature-classifier plugin (via "classify-sklearn") (45) using the pre-trained Silva 138.1 animal-distal-gut taxonomy classifier. For statistical analyses, the Phyloseq package (version 1.36.0) (46) was used to integrate the counts matrix, taxonomy and metadata. 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 (47) (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 was visualized with Ggplot2 (version 3.5.1).

Statistical analysis

All individual data points are shown and data are expressed as mean ± SEM, except for gene expression data, which is shown as geometric mean ± geometric SD. Statistical analyses were performed using GraphPad Prism 9.01 (GraphPad, La Jolla, California). 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 (α=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 eight weeks (Figure 1A). Both fiber and exercise training significantly decreased the body weight gain alone and in combination (Figure 1B, Suppl. Figure 1A). Both exercise training groups gained significantly more lean mass than the sedentary groups (Figure 1C, Suppl. Figure 1B), and both fiber and exercise training alone slowed fat mass gain compared to SED control, while the combination of fiber and exercise training led to fat mass loss (Figure 1D, Suppl. Figure 1C). No change in food intake was recorded throughout the study (Suppl. Figure 1D). Only RUN fiber had a lower weight of gWAT compared to SED (Figure 1E). Both exercise training groups decreased sWAT weight, with no changes between the sedentary groups with or without fiber supplementation (Figure 1F). In accordance with fat loss, RUN fiber also had decreased adipocyte size in sWAT compared to SED control, while any differences between other groups were not significant (Figure 1G, H). In line, RUN fiber was the only group with decreased plasma TG and TC levels compared to SED control, though both exercise training groups had decreased fasting plasma glucose compared to SED control (Figure 1I-K). Finally, despite fasting insulin levels being significantly decreased only in RUN fiber (Figure 1L), both fiber and exercise training alone and together decreased HOMA-IR compared to SED control (Figure 1M). Overall, both fiber and exercise training alone had positive effects on the general metabolic health, compared to SED control, while the combination of fiber and exercise training synergistically amplified these positive changes.

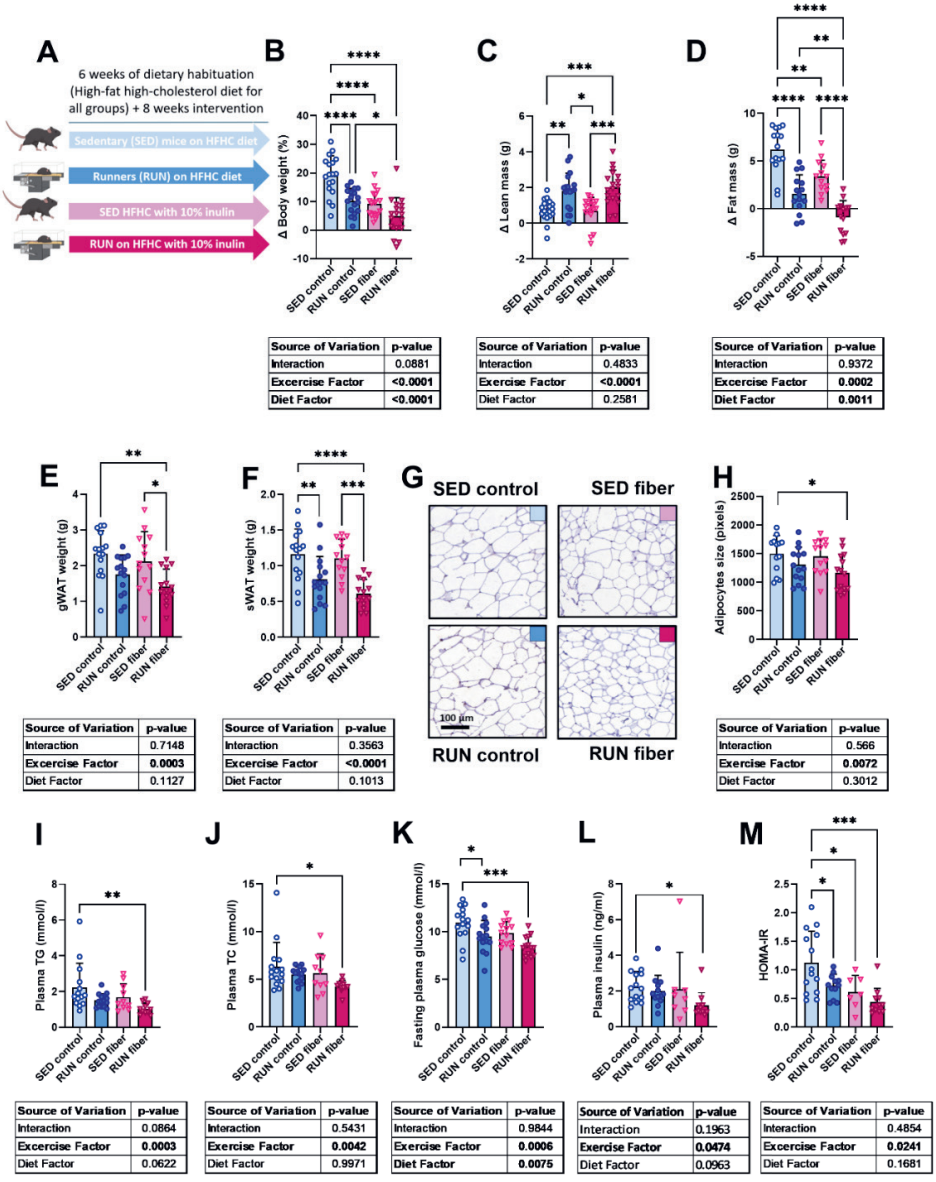


Figure 1. The effect of dietary fiber supplementation, exercise training and their combination on body composition and plasma parameters. After 6 weeks of dietary habituation to a high-fat high-cholesterol diet, male MASLD-prone APOE*3-Leiden.CETP mice either exercised, received 10% dietary inulin supplementation or a combination of both for 8 weeks (**A**). The change in body weight (**B**), lean mass (**C**) and fat mass (**D**) was measured over these 8 weeks. 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**, **H**). 4 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 mean ± SEM (n = 14-15), *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 according to two-way ANOVA followed by Sidak's multiple comparison test.

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 to SED control, although exercise training was overall beneficial (Exercise factor p=0.0007 in two-way ANOVA, Figure 2A). Both exercise training groups had a similar reduction in MASLD score, while SED fiber did not differ from SED control (Figure 2B,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), while fiber did not have any effect on the reduction of steatosis and steatosis-related parameters (Diet factor p<0.0001 in two-way ANOVA) (Figure 2D-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 to SED control, while RUN fiber did not (Figure 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 (Suppl. Figure 1E-H). Expression of *Hgmcr*, a gene encoding a protein involved in the endogenous cholesterol synthesis, was not changed either (Suppl. Figure 1H). Finally, the expression of genes encoding enzymes in the *de novo* lipogenesis pathway, *Fasn* and *Srebp1c*, was decreased similarly in all groups compared to SED control (Figure 2H). Taken together, both exercise training with and without fiber supplementation decreased the MASLD score and hepatic lipid accumulation, while fiber alone did not have an effect on MASLD amelioration.

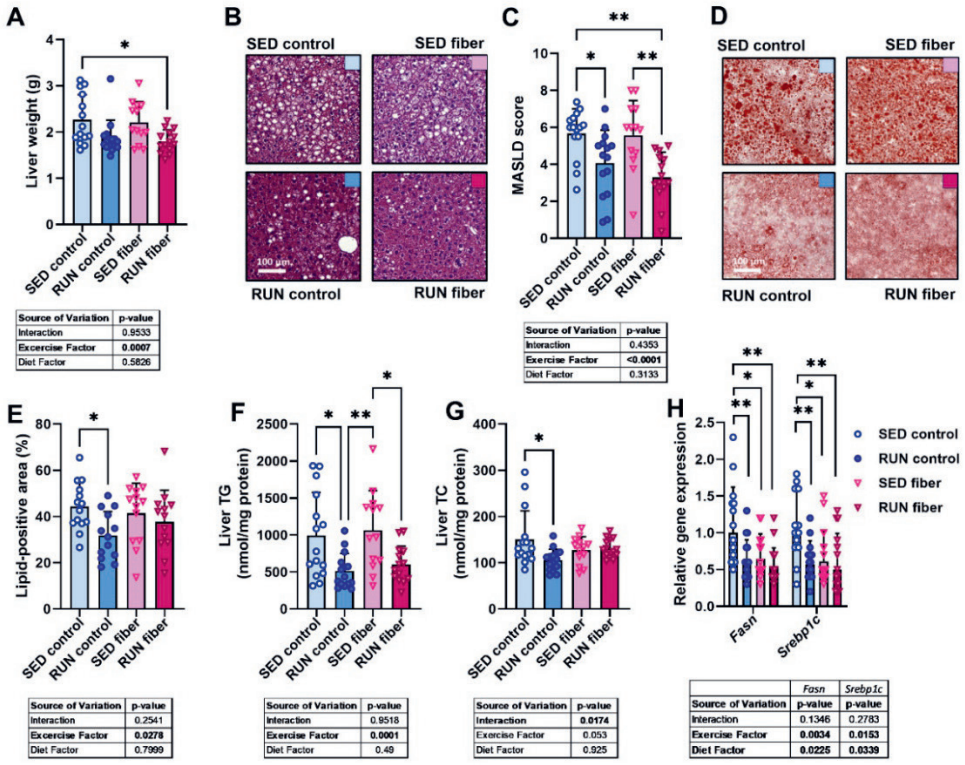


Figure 2. The effect of dietary fiber supplementation, exercise training and their combination on MASLD development. To investigate the effect of the interventions on MASLD development, mouse livers were collected and weighed (**A**). H&E stained liver sections (**B**) were used to determine the MASLD score (**C**), and ORO stained sections (**D**) were used to quantify lipid-positive area in the liver (**E**). Subsequently, liver lipids were isolated, and liver TG (**G**) 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 mean ± SEM (n = 14-15) for **A-G**, and as geometric mean ± geometric SD for **H**, *p < 0.05, **p < 0.01, according to two-way ANOVA followed by Sidak's multiple comparison test.

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 to SED control (p=0.052), while the F4/80-positive area was comparable between the two fiber groups and SED control (Figure 3A, B). Similarly, the expression of *Adgre1*, the gene encoding F4/80 in mice, and of *Tnfa,* encoding the pro-inflammatory cytokine TNF-α, were both decreased in RUN control compared to all other groups, while expression of *Col1a1*, a marker of fibrosis, was decreased only in RUN control compared to SED control (Figure 3C-E). The expression levels in both fiber groups remained unchanged compared to SED control (Figure 3C-E). These findings indicate that only exercise training without the addition of a high-fiber diet decreases liver inflammation under these experimental conditions.

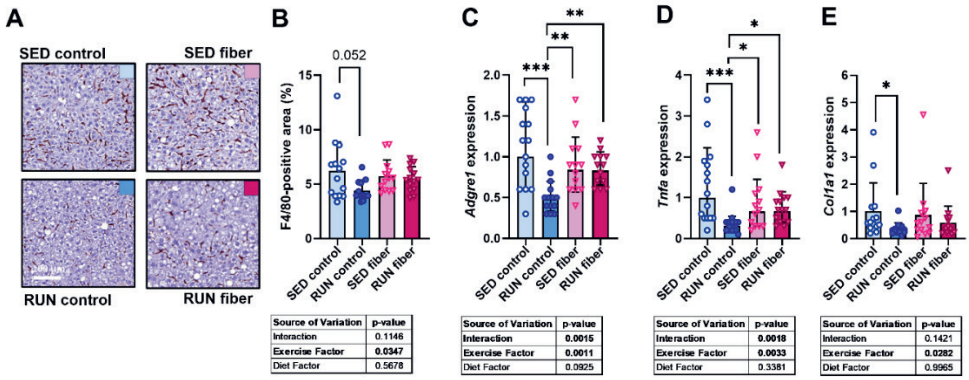
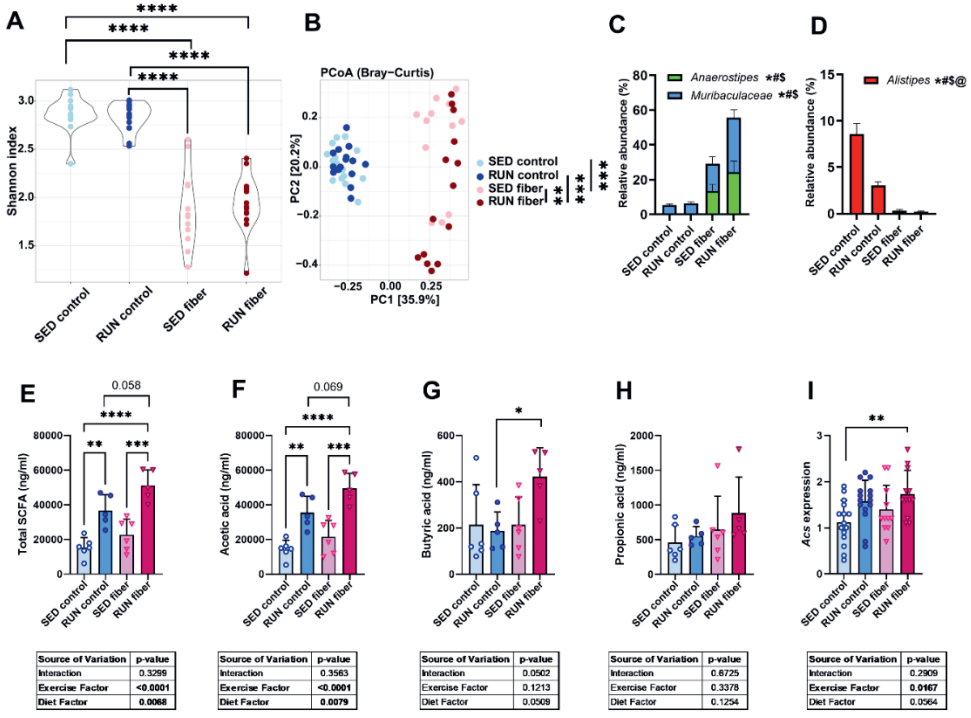


Figure 3. The effect of dietary fiber supplementation, exercise training and their combination on liver inflammation in MASLD. F4/80 staining (**A**) was used to quantify the amount of liver macrophages (**B**). Subsequently, the expression of *Adhesion G Protein-Coupled Receptor E1*, which encodes the murine macrophage marker F4/80 (**C**), *Tumor necrosis factor alpha* (*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 mean ± SEM (n = 14-15) for **B** and as geometric mean ± 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.

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 caecum content of the mice. Fiber diet decreased the α-diversity, a metric of the diversity within the sample (Figure 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 (Figure 4B). After multiple testing adjustments, there was no difference between SED control and RUN control in microbiota composition, while 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 (Figure 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* (Suppl. figure 2A). SED fiber also had a decreased abundance of *Firmicutes* (Suppl. figure 2A). On the family level, both fiber groups generally exhibited comparable changes. However, the *Lachnospiraceae* family was only increased in RUN fiber in comparison to all other groups (Suppl. Figure 2B). 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 (Figure 4C). *Muribaculaceae* was another notably increased genus in RUN fiber (Figure 4C). Together, these SCFA producers accounted for more than 55% of the total relative abundance in the RUN fiber group (Figure 4C). Their abundance was also increased to a lesser extent in SED fiber (Figure 4C). On the other hand, the abundance of the *Alistipes* genus was decreased in RUN control compared to the SED control, and was nearly completely depleted in both fiber groups (Figure 4D). Other genera changes were in line with previous observations, with fiber being the main driver of changes on each level (Suppl. Figure 3). 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 exercise training (Figure 4E). For instance, while 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 (Figure 4E). The RUN fiber SCFA concentration constituted a more than two-fold increase compared to 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 (Figure 4F). Curiously, butyric acid was only increased in the combination of fiber and exercise training group, while its levels in other groups remained similar to that of SED control (Figure 4G). No detectable difference in propionic acid levels was observed (Figure 4H). RUN fiber also showed a decreased concentration of 2-methylbutyric acid, while the levels of hexanoic acid, isobutyric acid, isovaleric acid and valeric acid were unchanged (Suppl. Figure 4). The SCFA levels positively correlated with *Muribaculaceae* abundance (Suppl. Figure 2 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 (Figure 4I). This correlated with the highest acetic acid levels in the portal vein in RUN fiber (Suppl. Figure 2F). 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.



Source of Variation	p-value
Interaction	0.3299
Exercise Factor	<0.0001
Diet Factor	0.0068

Source of Variation	p-value
Interaction	0.3563
Exercise Factor	<0.0001
Diet Factor	0.0079

Source of Variation	p-value
Interaction	0.0502
Exercise Factor	0.1213
Diet Factor	0.0509

Source of Variation	p-value
Interaction	0.6725
Exercise Factor	0.3378
Diet Factor	0.1254

Source of Variation	p-value
Interaction	0.2909
Exercise Factor	0.0167
Diet Factor	0.0564

Figure 4. The effect of dietary fiber supplementation, exercise training and their combination on gut microbiota composition and SCFAs abundance. To investigate changes in the gut microbiota composition, we conducted 16S bacterial sequencing. Alpha- (**A**) and beta-diversity (**B**) were measured. Differences between the groups in (**A**) were calculated with one-way ANOVA with Tukey post-hoc correction, while 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=14-15), where *- significant difference between RUN fiber vs SED control, # - SED fiber vs SED control, $ - RUN fiber vs RUN control, and & - RUN fiber vs SED fiber, @ - 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 mean ± SEM (n = 5-6) and as geometric mean ± geometric SD for **I** (n=14-15), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$, according to two-way ANOVA followed by Sidak's multiple comparison test.

Discussion

Dietary supplementation with fiber - in this study exclusively inulin - and exercise are both lifestyle interventions that are recommended for treating MASLD and other cardiometabolic 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 while this combination is beneficial for fat loss and glucose and lipid homeostasis, it does not further improve MASLD amelioration compared to 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, 48), as well as via an increase in muscle oxidative capacity (49). This would lead to more energy being used during exercise and would counteract hepatic lipid storage. Additionally, 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 (50-52). Finally, inulin by itself has been shown to be effective in reducing fasting glucose levels (53), which may be mediated by the suppression of resistin, a pro-inflammatory adipokine that modulates insulin function (54). 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. While generally SCFAs (specifically butyrate) are considered to be health-promoting, increased acetate levels in MASLD patients were previously seen to correlate with a worsening disease prognosis (55, 56). 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 (56) 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, while the combination treatment had no effect. Cholesterol may play a role in the development of hepatic inflammation (57, 58), 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 (59). 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 (55). Taken together, inulin itself and elevated acetate levels may counteract the reduction of liver inflammation achieved with exercise training.

Curiously, while 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. Firstly, 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. Secondly, fiber supplementation resulted in a β-diversity shift, with both fiber groups showing clear differences compared to the control groups. While 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 (60, 61), which can explain the increase in butyrate production in RUN fiber compared with other groups. As approximately 95% of butyrate is absorbed by colonocytes in the gut (50), 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 (62) and positively correlate with propionate (63) and acetate levels (64). While 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 remodelling 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 (65-68), 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 (66). In our study, the two most abundant bacterial genera, *Muribaculaceae* and *Anaerostipes*, have the capacity to convert lactate into acetate (64) and butyrate (69), 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 (70). 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 post-exercise SCFAs increase would also explain why exercise training or the combination treatment improved MASLD and other metabolic parameters, while the fiber diet by itself had a much smaller effect, as the production of SCFAs was not significantly increased in SED fiber compared to SED control.

Conclusion:

Overall, we show for the first time that while a combination of dietary fiber and exercise training is an effective strategy for weight loss, it does not synergistically contribute to MASLD amelioration compared to 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. Additionally, 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.

Acknowledgement: The authors thank Amanda C.M. Pronk, Salwa Afkir, Trea C.M. Streefland, Reshma A. Lalai, Chris van der Bent, Luna van Schaik and Hetty Sips for their excellent technical assistance.

Graphical abstract has been created in Biorender. Kovynev, A. (2025) https://BioRender.com/t00p596

Author contribution: A.K., P.C.N.R and M.S. designed the experiments; A.K, M.M.C., A.B. and M.S. collected and analyzed the data; A.K., Q.R.D. and M.S. drafted the manuscript; P.C.N.R. critically revised the manuscript.

Funding details: The project was supported by a LUF Grant to M.S. (LUF and Gratama-Stichting W223065-2-GSL). M.M.C. was supported by a Zimin Foundation SMTB Alumni Summer Research Program grant.

Disclosure statement: The authors declare no competing interests.

Data availability statement and data deposition: The 16S data can be accessed via the ZENODO database (10.5281/zenodo.13810371 and 10.5281/zenodo.13810601)

Declaration of generative AI and AI-assisted technologies in the writing process

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

Appendix:

Appendix 1: Supplementary figures and legends

Appendix 2: Dietary composition

Appendix 3: ImageJ scripts – can only be accessed online in the corresponding publication

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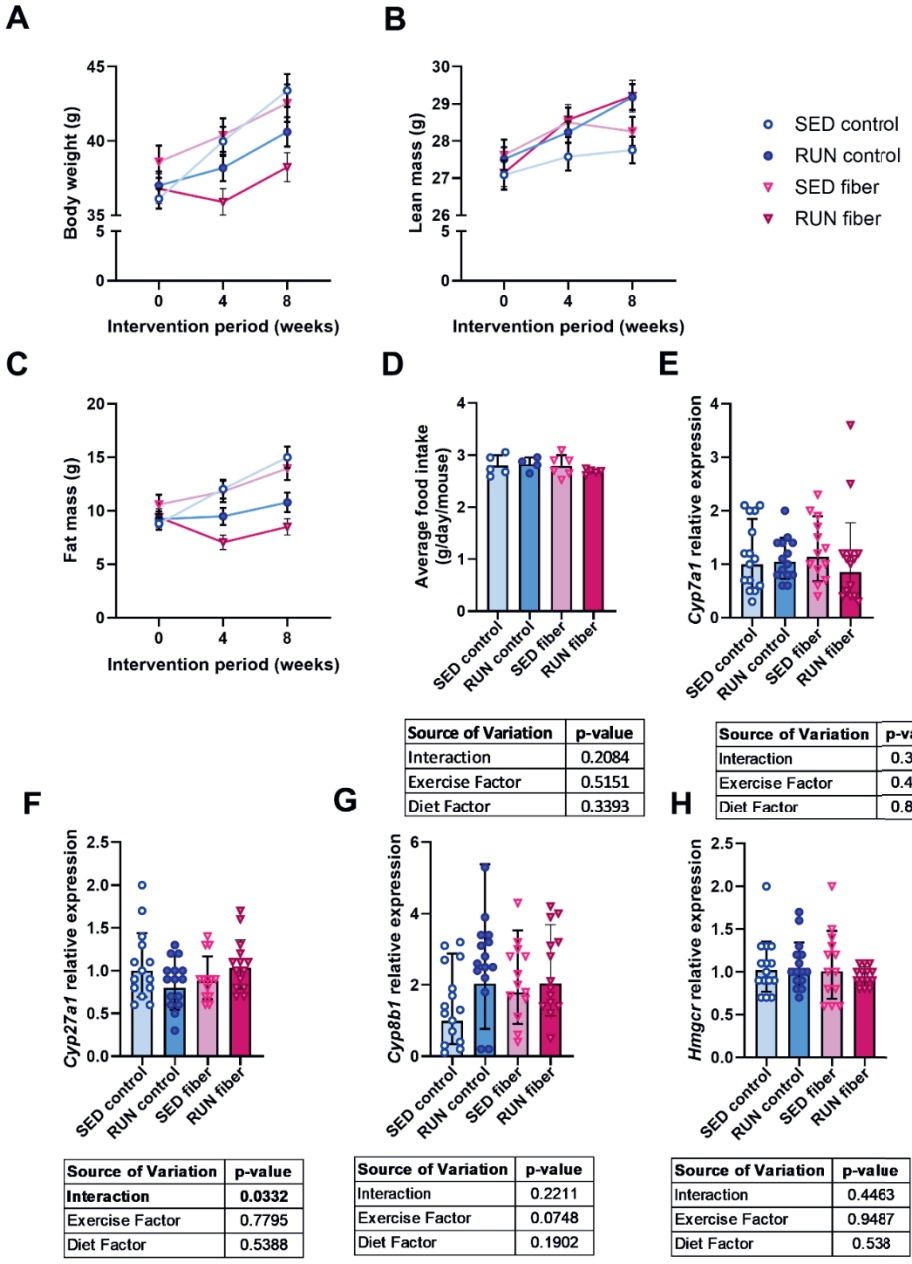
Supplementary file 1



Source of Variation	p-value
Interaction	0.2084
Exercise Factor	0.5151
Diet Factor	0.3393

Source of Variation	p-value
Interaction	0.3299
Exercise Factor	0.4182
Diet Factor	0.8367

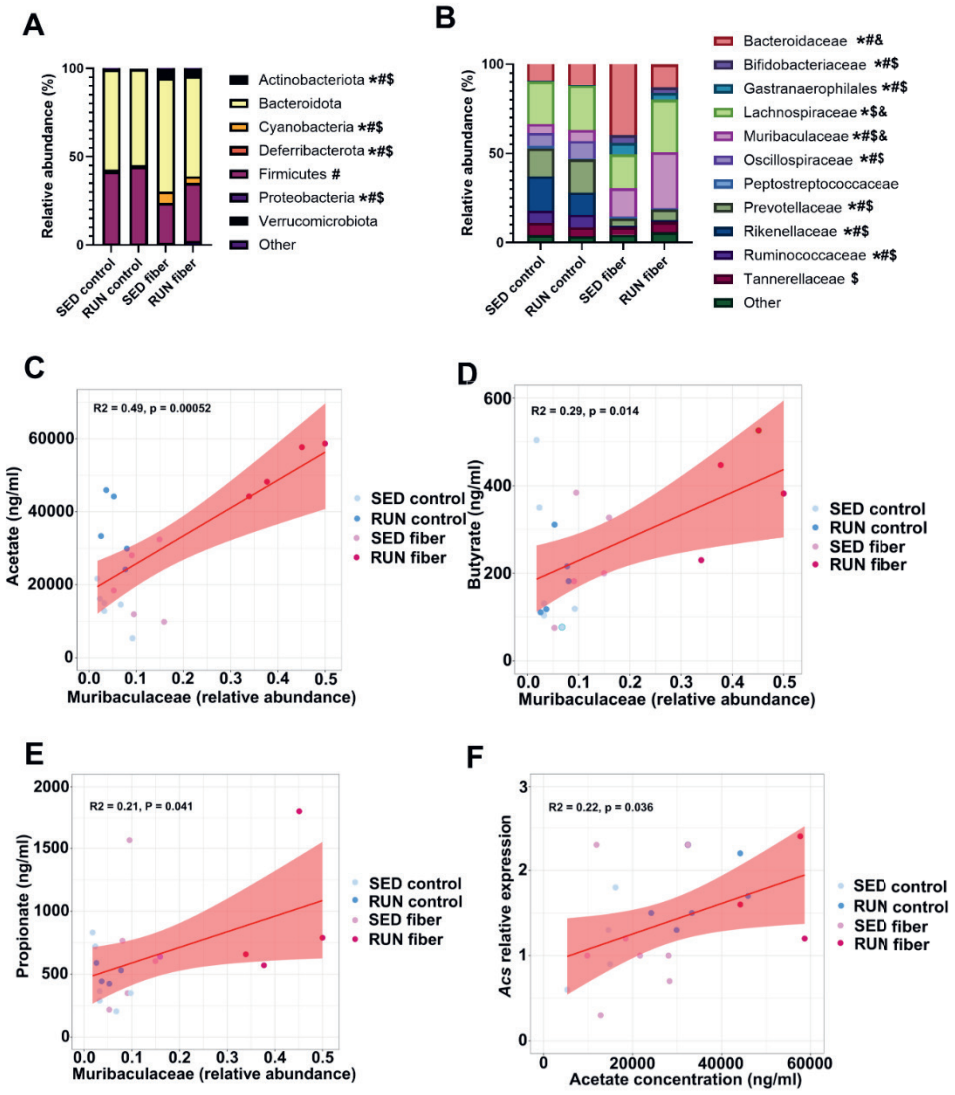
Source of Variation	p-value
Interaction	**0.0332**
Exercise Factor	0.7795
Diet Factor	0.5388

Source of Variation	p-value
Interaction	0.2211
Exercise Factor	0.0748
Diet Factor	0.1902

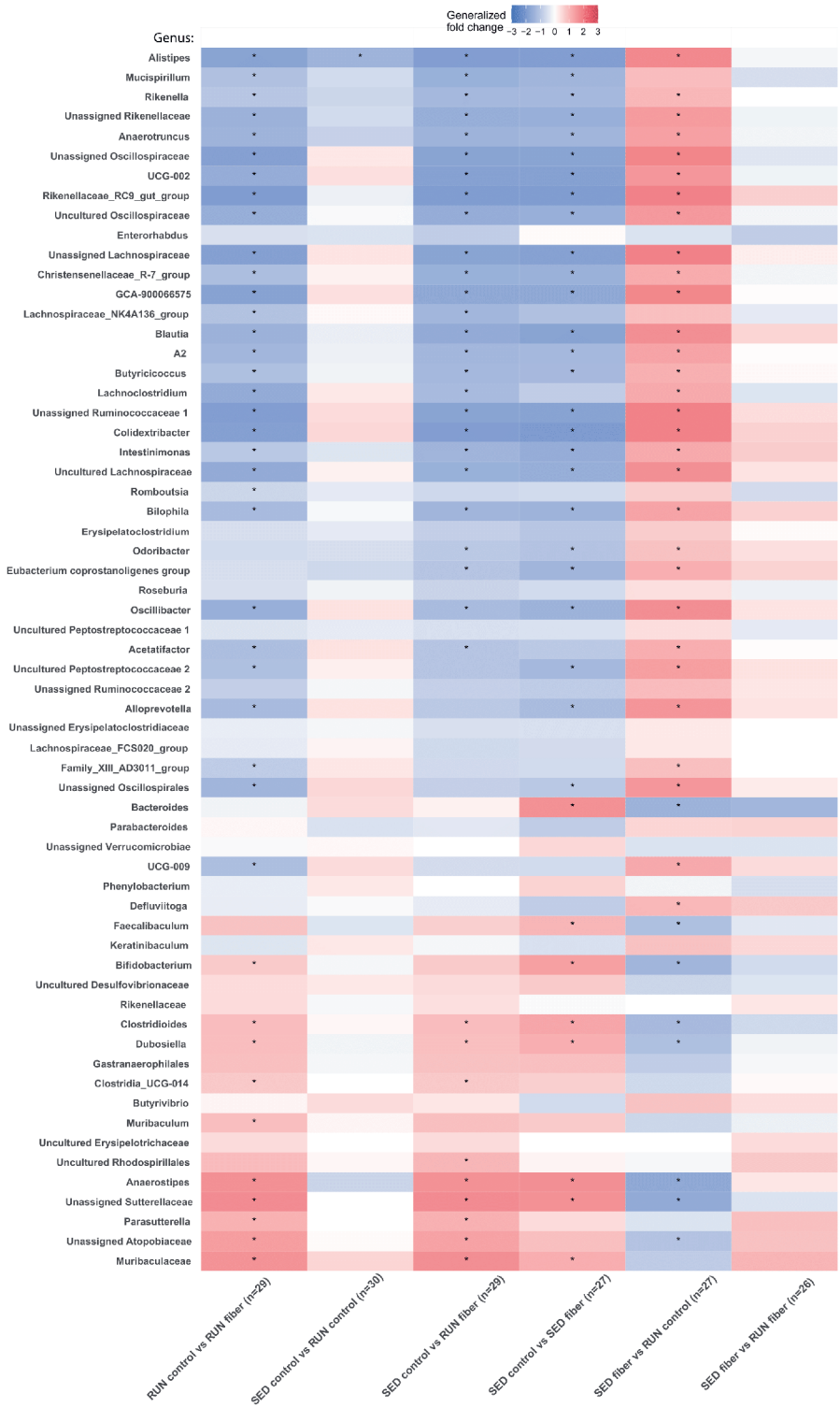
Source of Variation	p-value
Interaction	0.4463
Exercise Factor	0.9487
Diet Factor	0.538

Supplementary figure 1. The effect of dietary fiber supplementation, exercise training and their combination on body composition, insulin sensitivity and the bile acid synthesis pathways. Changes in overall body weight (**A**), fat mass (**B**), and

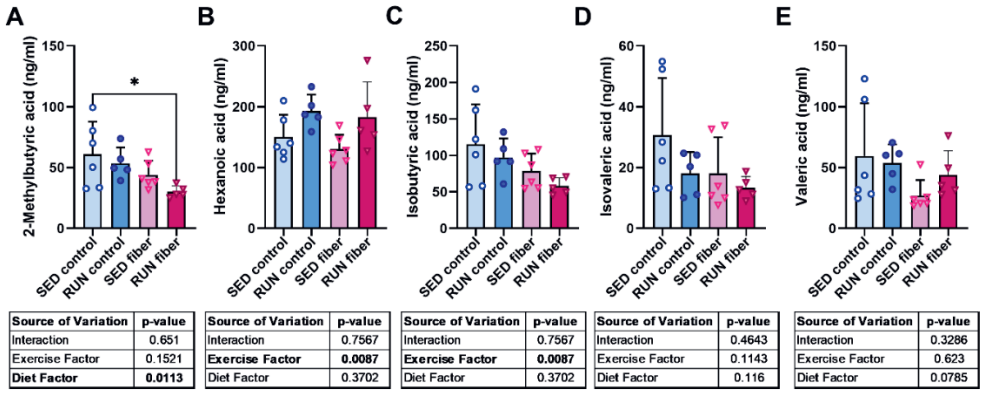
lean mass (**C**) were assessed every 4 weeks during the intervention period. Average daily food intake was assessed per cage and divided by the number of mice per cage (**D**). Hepatic gene expression of (**E**) *Cyp7a1*, (**F**) *Cyp27a1,* (**G**) *Cyp8b1* and (**H**) *Hmgcr* was measured. Data in **A-D** are presented as mean ± SEM and in **E-H** as geometric mean ± geometric SD (n=14-15 mice for all figures but **D**, where n=4-6 cages), *p < 0.05, **p < 0.01, ***p<0.001 according to two-way ANOVA followed by Sidak's multiple comparison test.



Supplementary figure 2. The effect of dietary fiber supplementation, exercise training and their combination on gut microbiota composition. To describe the overall changes in the gut microbiota, the abundance of the top 7 most abundant phyla (**A**) and top 11 most abundant families (**B**) are depicted. The correlations of the most increased genus *Muribaculaceae* with the abundance of acetate (**C**), butyrate (**D**) and propionate (**E**) in portal vein plasma were calculated. As acetate may be integrated into the Krebs cycle, acetate correlation with hepatic *Acs* expression was also assessed (**F**) Data in **A-B** is presented as an average value per group, (n=14-15), where *- significant difference between RUN fiber vs SED control, # - SED fiber vs SED control, $ - RUN fiber vs RUN control, and & - RUN fiber vs SED fiber, according to Wilcoxon test, adjusted for multiple testing. Pearson correlation index was used for **C-F** (n=5 per group).


Generalized fold change
−3 −2 −1 0 1 2 3
Genus:
Alistipes
Mucispirillum
Rikenella
Unassigned Rikenellaceae
Anaerotruncus
Unassigned Oscillospiraceae
UCG-002
Rikenellaceae_RC9_gut_group
Uncultured Oscillospiraceae
Enterorhabdus
Unassigned Lachnospiraceae
Christensenellaceae_R-7_group
GCA-900066575
Lachnospiraceae_NK4A136_group
Blautia
A2
Butyricicoccus
Lachnoclostridium
Unassigned Ruminococcaceae 1
Colidextribacter
Intestinimonas
Uncultured Lachnospiraceae
Romboutsia
Bilophila
Erysipelatoclostridium
Odoribacter
Eubacterium coprostanoligenes group
Roseburia
Oscillibacter
Uncultured Peptostreptococcaceae 1
Acetatifactor
Uncultured Peptostreptococcaceae 2
Unassigned Ruminococcaceae 2
Alloprevotella
Unassigned Erysipelatoclostridiaceae
Lachnospiraceae_FCS020_group
Family_XIII_AD3011_group
Unassigned Oscillospirales
Bacteroides
Parabacteroides
Unassigned Verrucomicrobiae
UCG-009
Phenylobacterium
Defluviitoga
Faecalibaculum
Keratinibaculum
Bifidobacterium
Uncultured Desulfovibrionaceae
Rikenellaceae
Clostridioides
Dubosiella
Gastranaerophilales
Clostridia_UCG-014
Butyrivibrio
Muribaculum
Uncultured Erysipelotrichaceae
Uncultured Rhodospirillales
Anaerostipes
Unassigned Sutterellaceae
Parasutterella
Unassigned Atopobiaceae
Muribaculaceae
RUN control vs RUN fiber (n=29)
SED control vs RUN control (n=30)
SED control vs RUN fiber (n=29)
SED control vs SED fiber (n=27)
SED fiber vs RUN control (n=27)
SED fiber vs RUN fiber (n=26)

Supplementary figure 3. **The effect of dietary fiber supplementation, exercise training and their combination on gut microbiota composition of genera, here shown as comparison between the groups.** To describe all changes on the genus level, a heatmap of genera that tended to be changed (p.adj < 0.15) in at least one comparison was generated. "n=" indicates how many samples were used for the comparison. Comparisons where p.adj < 0.05 are marked with *, according to Wilcoxon test, adjusted for multiple testing.



Source of Variation	p-value
Interaction	0.651
Exercise Factor	0.1521
Diet Factor	**0.0113**

Source of Variation	p-value
Interaction	0.7567
Exercise Factor	**0.0087**
Diet Factor	0.3702

Source of Variation	p-value
Interaction	0.7567
Exercise Factor	**0.0087**
Diet Factor	0.3702

Source of Variation	p-value
Interaction	0.4643
Exercise Factor	0.1143
Diet Factor	0.116

Source of Variation	p-value
Interaction	0.3286
Exercise Factor	0.623
Diet Factor	0.0785

Supplementary figure 4. The effect of dietary fiber supplementation, exercise training and their combination on SCFA concentration in portal vein plasma. Levels of 2-methylbutyric acid (**A**), hexanoic acid (**B**), isobutyric acid (**C**), isovaleric acid (**D**) and valeric acid (**E**) were measured in portal vein plasma. Data are presented as mean ± SEM (n=5-6), *p < 0.05, according to two-way ANOVA followed by Sidak's multiple comparison test.

Supplementary file 2:

Composition of diets used in the study:

Chow diet

Standard chow diet that mice were fed before the start of the experiment can be found here:

https://www.sds-diets.com/sds-wAssets/docs/rodent/sds_rm3-p-_ds.pdf

Control diet

For the control diet, we used the modified Base high-fat diet D12492 from ssniff Spezialdiäten GmbH, Soest, Germany.

The base diet can be found here:

https://www.ssniff.com/documents/03-03%20%20Purified%20DIO%20&%20Controls_v.pdf

The base diet has been modified with the addition of 1% cholesterol

The final diet composition was as follows:

Crude nutrients	%
Crude protein	24.4
Crude fat	34.6
Crude fiber	6
Crude ash	5.3
Astarch	0.1
Sugar	8.4
Cholesterol	1

Fiber diet

For the fiber diet, inulin was supplemented to constitute 10% of the diet. As a result, in total, the fiber diet had 1% less of maltodextrin, 1% less of sucrose, 5% less of cellulose and 7% more of inulin compared to control diet.

The final diet composition was as follows:

Crude nutrients	%
Crude protein	24.4
Crude fat	34.6
Crude fiber	10
Crude ash	5.3
Astarch	0.1
Sugar	7.8
Cholesterol	1