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Citation
Kovynev, A., Ying, Z. X., Zhang, S., Olgiati, E., Lambooij, J. M., Visentin, C., … Schönke, M. (2024). Timing matters: late, but not early, exercise training ameliorates MASLD in part by modulating the gut-liver axis in mice. *Journal Of Pineal Research*, *76*(8). doi:10.1111/jpi.70003

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ORIGINAL ARTICLE OPEN ACCESS

Timing Matters: Late, but Not Early, Exercise Training Ameliorates MASLD in Part by Modulating the Gut-Liver Axis in Mice

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Received: 17 September 2024 | **Revised:** 10 October 2024 | **Accepted:** 21 October 2024

Funding: This work was supported by the Novo Nordisk Foundation (grant NNF18OC0032394 to M.S.), The Netherlands Cardiovascular Research Initiative CVON-GENIUS-2 (grant to P.C.N.R.), the Chinese Scholarship Council (grants to Z.Y. and S.Z.). A.K. is supported by a PhD grant from Leiden University Medical Center (to M.S.).

Keywords: circadian rhythms | exercise | gut microbiota | MASLD | mouse model

ABSTRACT

Metabolic dysfunction-associated steatotic liver disease (MASLD) affects two billion people worldwide and is currently mostly treatable via lifestyle interventions, such as exercise training. However, it is unclear whether the positive effects of exercise are restricted to unique circadian windows. We therefore aimed to study whether the timing of exercise training differentially modulates MASLD development. Twenty weeks old male APOE*3-Leiden.CETP mice were fed a high fat-high cholesterol diet to induce MASLD and treadmill-trained for 1 h five times per week for 12 weeks either early (ZT13; E-RUN) or late (ZT22; L-RUN) in the dark phase while corresponding sedentary groups (E-SED and L-SED) did not. Late, but not early exercise training decreased the MASLD score, body weight, fat mass, and liver triglycerides, accompanied by an altered composition of the gut microbiota. Specifically, only late exercise training increased the abundance of short-chain fatty acid-producing bacterial families and genera, such as *Akkermansia, Lachnospiraceae*, and *Rikenella*. To assess the role of the gut microbiota in training-induced effects, the study was repeated and trained (ZT22 only, RUN) or sedentary mice (SED) served as fecal donors for sedentary recipient mice (RUN FMT and SED FMT). Fecal microbiota transplantation reduced liver weight and plasma triglycerides in RUN FMT compared to SED FMT and tended to lower the MASLD score and liver triglycerides. Timing of exercise training is a critical factor for the positive effect on MASLD in this preclinical model, and the effect of late exercise is partially mediated via the gut-liver axis.

1 | Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD, formerly known as NAFLD) is a major public health burden that affects more than 30% of the global population, and its prevalence is predicted to increase further in the future [1, 2]. MASLD is characterized by a prolonged accumulation of lipid droplets in the liver, which is generally a result of excessive caloric intake [3]. This accumulation induces the release of pro-inflammatory signals, promoting an influx of immune cells into the liver and an advancement of the disease to metabolic dysfunction-associated steatohepatitis (MASH), which may lead to liver fibrosis. Gut-liver

Artemiy Kovynev and Zhixiong Ying contributed equally.

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axis dysfunction has recently emerged as an additional MASLD contributor, with changes in the composition and function of the gut microbiota leading to higher energy harvesting and lipid absorption and an increased influx of pro-inflammatory molecules into the liver [4]. Liver steatosis and MASH are reversible, but pharmaceutical treatment options are currently limited, with the first anti-MASLD treatment having been conditionally approved by the USA Food and Drug Administration only this year [5]. As a result, the most common viable treatment strategies are lifestyle interventions, such as dietary changes and increasing physical activity. It is crucial to optimize such interventions to maximize their effectivity [6].

One potential way of maximizing intervention effectivity is the choice of an appropriate circadian window for exercise. Nearly all processes in the body are controlled by circadian rhythms [7]. In addition to the central clock in the hypothalamus, organs have their own peripheral circadian clocks to optimize their function [8]. MASLD dampens the circadian rhythmicity of cellular processes in the gut and the liver, leading to increased metabolic disturbances, while exercise training can contribute to "fine-tuning" of the clock, at least in skeletal muscle [8, 9] and liver [10, 11]. However, it remains unclear what the optimal training time is for the treatment and prevention of MASLD. In patients with obesity and type 2 diabetes, late exercise training improves glycemic control, whereas early exercise training causes unfavorable spikes in glycemia [12]. Epidemiological studies show that increased afternoon physical activity decreases the risk of cardiometabolic diseases compared to morning physical activity in the Netherlands Epidemiology of Obesity cohort [13], even though the contrary was observed in a comparable sedentary and overweight population from the UK Biobank [14]. In mice, we have previously shown that only late, but not early exercise training reduces the development of atherosclerosis [15], a disease closely related to MASLD in pathogenesis. Surprisingly, in mild MASLD, early exercise training promotes the influx of pro-inflammatory cells into the liver, which is normally observed with disease progression, while late exercise training does not [16]. Additionally, we identified an enrichment of potentially health-promoting gut bacteria uniquely with late exercise training in mice [15].

Disturbances in the gut-liver axis have been implicated in multiple cardiometabolic diseases [17]. In humans, Western diet decreases the microbial diversity in the gut and reduces the abundance of *Ruminococcaceae* and *Bifidobacterium*, two bacterial taxa associated with decreased liver fibrosis [17]. Western diet also increases bacterial ethanol production, leading to higher energy harvesting and, as a result, increases calorie absorption from the consumed food [17, 18]. Furthermore, these changes lead to a leakier gut barrier due to impaired tight junctions between enterocytes [19]. As a result, more microbial molecules such as lipopolysaccharide (LPS) can reach the bloodstream and increase hepatic and systemic inflammation [17, 19]. Finally, a high-fat diet decreases the amount of short-chain fatty acid (SCFA)-producing bacteria and specifically butyrate producers, which is generally associated with an unfavorable disease outcome [17]. Consequently, addressing gut dysbiosis in MASLD via lifestyle interventions may be a step forward to ameliorating disease progression.

In the current study, we investigated the influence of early and late active period exercise training on the amelioration of MASLD in high fat-high cholesterol (HFHC) diet-fed male APOE*3-Leiden.CETP mice, a model mimicking human MASLD development [20].

2 | Materials and Methods

2.1 | Animal Experiments

Male APOE*3-Leiden.CETP mice were bred as previously described [21]. In experiment 1, mice at the age of 20–26 weeks were group housed (two to four mice per cage) under standard conditions (22°C, 12/12 h light/dark cycle; the light phase started at *Zeitgeber* time/ZT 0) with ad libitum access to water and a high-fat high-cholesterol (HFHC) diet (60 kJ% fat + 1% w/w cholesterol; D12492, ssniff Spezialdiäten GmbH, Soest, Germany). Cages were enriched with cardboard rolls, nesting material and a wooden stick for chewing. After 3 weeks 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, in total 72 animals) using RandoMice version 1.0.9 [22]. Two groups exercised on a treadmill at the same running speed five consecutive days per week (Monday to Friday) for 12 weeks either at ZT13 ("early runners," E-RUN) or at ZT22 ("late runners," L-RUN) for 1 h, while early and late sedentary mice (E-SED and L-SED, respectively) 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 fasted for 4 h and killed at ZT18; therefore, E-RUN and L-RUN mice were killed 28 and 19 h after their last exercise training, respectively. The same ZT time of sacrifice for all groups was chosen to eliminate the circadian oscillations bias, with ZT18 being between the two training timepoints. Five mice per group received an oral gavage of FITC-Dextran 3 h into the fasting period (see section "Intestinal permeability measurement," Supplementary information), and another five mice per group received an intravenous injection of glycerol tri[^{3}H]oleate ([^{3}H]triolein; [^{3}H]TO)-labeled TG-rich lipoprotein (TRL)-like particles) together with [^{14}C]deoxyglucose after the fast (see section "In vivo organ uptake of TRL-mimicking particles and glucose," Supplementary document 2). The remaining eight mice per group did not receive any treatment, and their immune cells were isolated from liver and used for flow cytometry analysis (see section "Liver immune cell isolation and flow cytometry" Supplementary document 2). The mice used for each treatment were matched by body mass, fat mass and plasma TG levels. All mice were killed by CO_2 asphyxiation before portal vein blood (only in mice used for immune cell isolation) and heart puncture blood was collected, and the animals were perfused with PBS (ice-cold for all mice except the ones used for flow cytometry analysis) and multiple organs (liver, gonadal white adipose tissue (gWAT), subcutaneous white adipose tissue (sWAT), interscapular brown adipose tissue (iBAT), quadriceps, tibialis anterior (TA) muscle, gastrocnemius muscle, as well as cecum and cecum content) were collected. Four mice were excluded due to tumors/cysts in different parts of the body.

In experiment 2 (timeline overview in Figure S6A), male APOE*3-Leiden.CETP mice at the age of 10 to 14 weeks were housed under the same conditions and fed with the same HFHC diet as in experiment 1. After 3 weeks of dietary acclimatization, mice were

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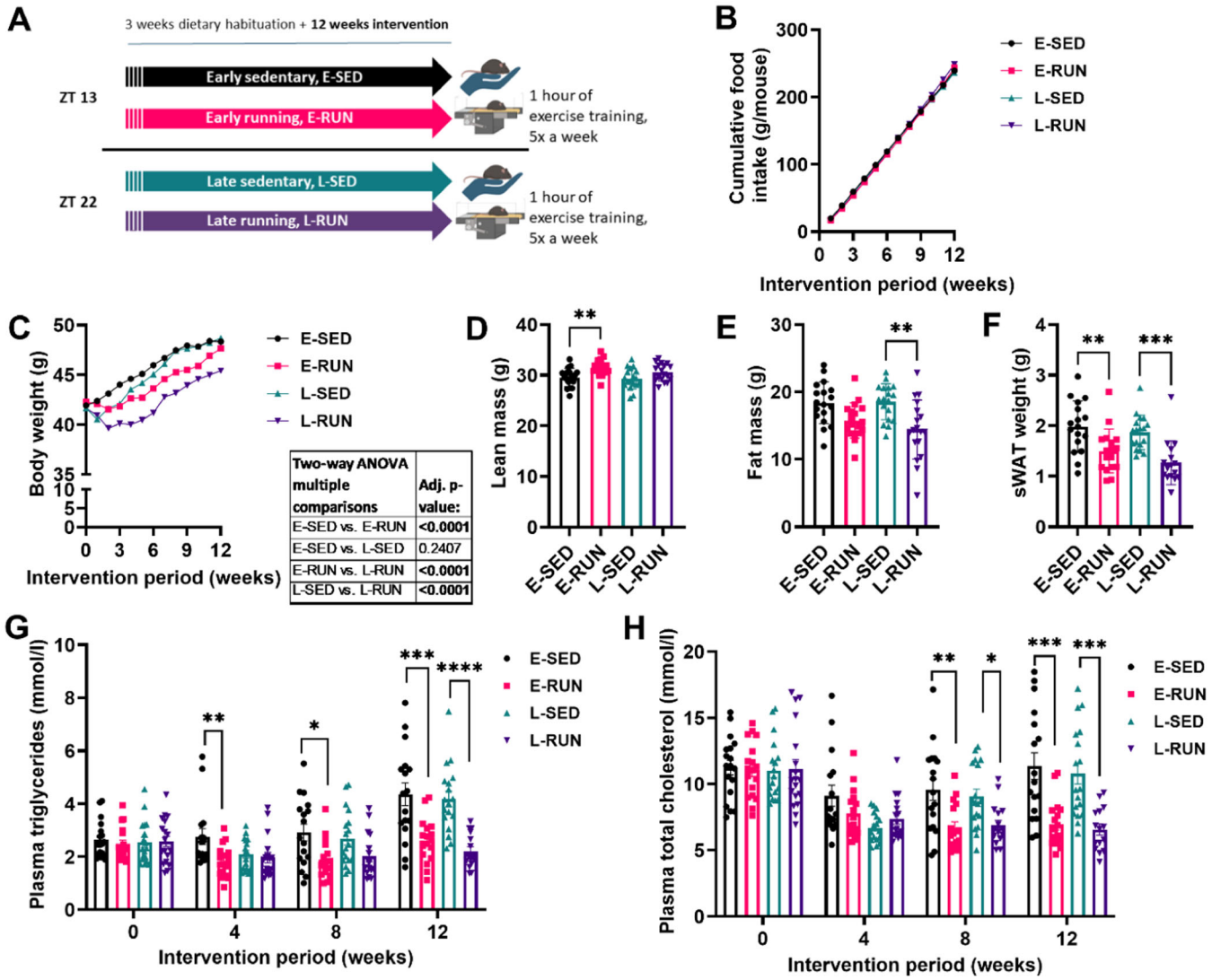

FIGURE 1 | Effect of early and late exercise training on body composition and plasma lipids. (A) MASLD-prone male APOE*3-Leiden.CETP mice on a HFHC diet were treadmill-trained five times per week either in the early (Zeitgeber time/ZT 13) or late (ZT 22) dark phase for 12 weeks. (B) Cumulative food intake and (C) body weight gain were assessed throughout the study, and (D) lean mass, (E) fat mass and (F) subcutaneous white adipose tissue (sWAT) mass were measured at endpoint. (G) Plasma triglycerides and (H) total cholesterol levels were assessed throughout the study. Data are presented as mean ± SEM ($n = 16$–18), $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ according to one-way ANOVA (D–F) or two-way ANOVA (B, C, G, H) followed by the Tukey multiple comparison test.

divided into fecal microbiota donors and fecal microbiota recipients. Donors either exercised on the treadmill five times per week for twelve weeks (RUN group) or were handled (SED group) at ZT22 ($n = 20$ per group, in total 80 animals) as described. From 4 weeks of training onwards, fresh feces were collected from the donors and pooled per group for fecal microbiota transplantation (FMT) three times per week for 8 weeks, leading to the recipients being divided into a RUN FMT group and a SED FMT group ($n = 20$ per group) (Figure 5A). Two weeks before the FMT, recipients were subjected to antibiotics treatment for 2 weeks to diminish the endogenous gut microbiota and improve FMT engraftment (see sections "Antibiotic treatment" and "fecal microbiota transplantation," Supplementary document S2). All mice were killed at ZT18 by CO_2 asphyxiation before portal vein blood and heart puncture blood was collected, the animals were perfused with ice-cold PBS, and multiple organs were collected as described above.

All procedures are described in detail in Supplementary document S2. We used the ARRIVE [23] checklist when writing our report.

2.2 | Ethics Statement

All animal experiments were carried out according to the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and were 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 (Protocols No. PE.21.002.029 and PE.21.002.057 for the first and second animal experiment, respectively).

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2.3 | Statistical Analysis

All individual data points are shown and data are expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism 9.01 (GraphPad, La Jolla, California) and one-way ANOVAs followed by Tukey's multiple comparisons test where appropriate. The data were tested for normality and all data sets were normally distributed. 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 $p < 0.0001$ (****).

3 | Results

3.1 | Only Late, but Not Early Exercise Training Reduces Body Weight Gain

To investigate the impact of timed exercise training on the development of MASLD, male APOE*3-Leiden.CETP mice were fed a HFHC diet and exercise-trained five times per week for 12 weeks either at ZT13 (E-RUN) or ZT22 (L-RUN), equivalent to the human morning and evening (Figure 1A). Exercise training at either ZT13 or ZT22 did not affect food intake (Figure 1B; Figure S1A,B). E-RUN gained less body weight from week 5 to 10 but caught up by week 12 as compared to E-SED (Figure 1C). On the other hand, late exercise training attenuated body weight gain throughout the study (Figure 1C). By week 12, E-RUN had a higher lean mass than E-SED, while there was no difference between L-RUN and L-SED (Figure 1D; Figure S1C). Both early and late exercise training led to a smaller fat mass by week 12 as compared to their sedentary counterparts. However, the difference reached statistical significance only with late exercise training (Figure 1E; Figure S1D). In line, the weight of sWAT was lower in both exercise training groups (Figure 1F). Both early and late exercise training decreased levels of plasma TG and TC as compared to their respective sedentary controls (Figure 1G,H). Taken together, both early and late exercise training showed positive effects on overall health, but late exercise training more effectively attenuated body weight gain.

3.2 | Only Late, but Not Early Exercise Training Alleviates Liver Steatosis

To evaluate the effects of early and late exercise training on the amelioration of MASLD, we next assessed liver health and lipid accumulation. L-RUN but not E-RUN showed smaller liver sizes as compared to their corresponding sedentary counterparts (Figure 2A). Strikingly, this late exercise-specific benefit was also observed when assessing the MASLD score, where L-RUN showed a lower score than L-SED while the scores of E-RUN did not differ from E-SED (Figure 2B,C). This was mainly explained by less macrosteatosis and hepatocyte hypertrophy in L-RUN (Figure S2A–C). These results were further supported by biochemical measurements showing that hepatic TG content was only lowered by late, but not early exercise training (Figure 2D). Hepatic cholesterol content, on the other hand, was decreased in both E-RUN and L-RUN compared to the corresponding SED groups (Figure 2E). To investigate the underlying cause for the improved hepatic steatosis with late exercise training, we quantified the organ uptake of lipids and glucose using [^{3}H]TO-labeled TRL-like particles and [^{14}C]deoxyglucose. Here, only late exercise training tended to decrease the uptake of [^{3}H]TO and [^{14}C]deoxyglucose by the liver (Figure S2E,F). We did not observe differences in the uptake of these tracers by adipose tissue or skeletal muscle (Figure S3A–H). We next measured the expression of hepatic genes related to fatty acid and bile acid metabolism. No changes between groups were observed in the expression of genes associated with *de novo* lipogenesis (*Srebp1c* and *Fasn*), fatty acid uptake (*Cd36*) and oxidation (*Cpt1a*) (Figure 2F). The expression of *Cyp7a1* and *Cyp7b1*, encoding key enzymes in the primary bile acid synthesis pathways, was upregulated by only late training and by both late and early training, respectively (Figure S2G–L). Despite the unchanged lipid and glucose uptake by adipose tissue and unchanged adipocyte sizes (Figure S3I), both exercise groups showed fewer crown-like structures in sWAT, a marker of tissue inflammation (Figure S3J). Finally, because of the absence of changes in tracer uptake by muscle, we measured the abundance of mitochondrial respiratory complexes in the skeletal muscles to quantify muscle oxidative capacity in the mice subjected to the differently timed exercise regimens. There was no change in the protein abundance between the groups (Figure S3K–Q). Taken together, only late exercise training ameliorated liver steatosis in conjunction with changes in hepatic energy uptake.

3.3 | Exercise Training Regardless of Timing Attenuates Inflammation and Fibrosis in the Liver

Next, we investigated the inflammatory and fibrogenic processes that drive MASLD progression. As determined in H&E-stained liver sections and similarly to attenuated inflammation in white adipose tissue, the inflammation score was similarly lowered in E-RUN and L-RUN compared to their sedentary counterparts (Figure S2D). In line, the hepatic abundance of F4/80 protein, a marker of murine macrophages, was lowered in both exercise training groups (Figure 3A,B). The expression of *Adgre1*, which encodes F4/80, only tended to decrease upon late exercise training, while in line with the previous observations, the mRNA expression level of *Tnfa*, encoding a main inflammatory cytokine secreted by macrophages, was decreased in both exercise training groups (Figure 3C). The expression of *Il1b*, another pro-inflammatory cytokine, was not affected (Figure 3C). This anti-inflammatory effect of exercise was further supported by flow cytometry data (Figure S4A–X) showing that both early and late exercise decreased the number of CD11c$^+$ cells, a marker of MASLD progression, in a subset of macrophages (Figure S4T,V,X). To assess the impact of the exercise training on fibrosis development, the hepatic collagen content was determined via histological staining and found to be decreased in both E-RUN and L-RUN compared to their corresponding sedentary controls (Figure 3D,E). In line, the gene expression of *Col1a1*, which encodes the major component of type I collagen, was found to be downregulated in both exercise training groups (Figure 3C). Together, these results demonstrate that exercise training independently of timing attenuates inflammation and fibrogenesis in the liver.

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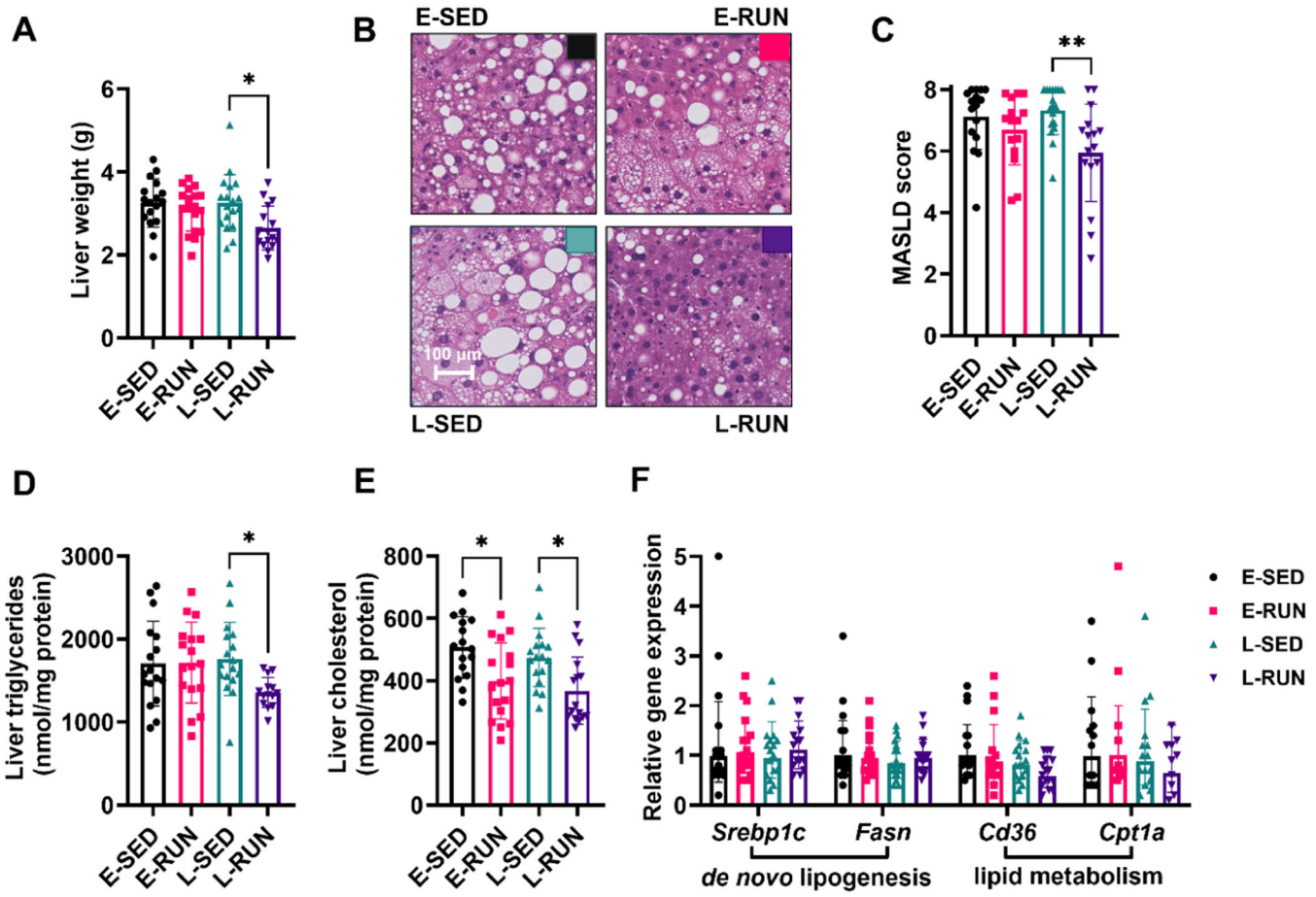

FIGURE 2 | Effect of early and late exercise training on liver steatosis. At the end of the 12-week experiment, (A) liver weight was measured and (B,C) MASLD score was determined on H&E stained liver sections. The liver content of (D) triglycerides and (E) total cholesterol was assessed, and (F) the expression of genes related to lipid metabolism were measured in the liver. *Srebp1c*, sterol regulatory element-binding protein 1 c; *Fasn*, fatty acid synthase; *Cd36*, cluster of differentiation 36; *Cpt1a*, carnitine palmitoyltransferase 1α. Data are presented as mean ± SEM in A, B and D, E or as geometric mean ± geometric SD in F ($n = 16–18$). *$p < 0.05$, ****$p < 0.0001$ according to one-way ANOVA followed by Tukey's multiple comparison test.

3.4 | Only Late, but Not Early Exercise Training Reshapes Gut Microbiota Composition

Since MASLD is modulated by gut dysbiosis, we next investigated whether the underlying mechanisms of the effect of late exercise training on MASLD are associated with microbiota changes. For this, we performed 16S sequencing of mouse cecum content. Neither early nor late exercise training changed the α-diversity, a measure of bacterial community richness (Suppl. Figure 5A). Late exercise training, however, led to a shift in microbial composition as reflected by a different β-diversity of L-RUN compared to L-SED, while this was not seen for E-SED and E-RUN (Figure S5B). To further investigate what constitutes this microbial shift, we investigated the changes in the relative abundance of bacteria on phylum, family and genus levels. On the phylum level, *Firmicutes* increased significantly in L-RUN compared to L-SED, with no other significant changes (Figure 4A). On the family level, *Clostridia_vadinBB60_group* increased fourfold in L-RUN compared to both L-SED and E-RUN (Figure S5C). On the genus level, both exercise training groups increased the relative abundance of *Lactobacillus* (Figure 4B). Compared to both L-SED and E-RUN, L-RUN decreased the abundance of *Alistipes* (Figure S5D), while increasing the abundance of *Rikenella* and *Lachnospiraceae UCG-006* (Figure 4B). L-RUN also had an increased abundance of *Acetatifactor* and *Akkermansia* compared to L-SED (Figure 4B). These enriched species are known SCFA producers.

Since 16S sequencing only determines the relative abundance of bacteria with few functional insights and the current murine gut databases leave a large number of unassigned taxa, we conducted shotgun metagenomics sequencing on the combined group of E-SED and L-SED (further referred to as SED) and E-RUN and L-RUN. In agreement with the 16S sequencing data, shotgun metagenomics sequencing revealed a strong shift in β-diversity in only L-RUN compared to both E-RUN and SED (Figure 4C,D), confirming that overall only late exercise training led to changes in the gut microbiota composition. On the genus level, similarly to the 16S sequencing results, exercise training increased the abundance of *Lactobacillus* which only reached significance for L-RUN (Figure 4E). In line with the 16S sequencing, the abundance of *Alistipes* was dramatically suppressed in L-RUN, with E-RUN following a similar trend, while *Blautia* was only reduced by late exercise training (Figure 4F). Curiously, we found two species, *Lachnospiraceae species i.s.* and *Ruminococcaceae species i.s.* (Figure 4G), constituting 1% of the overall relative abundance, which were only increased in L-RUN. Despite reshaping the gut microbiota, late

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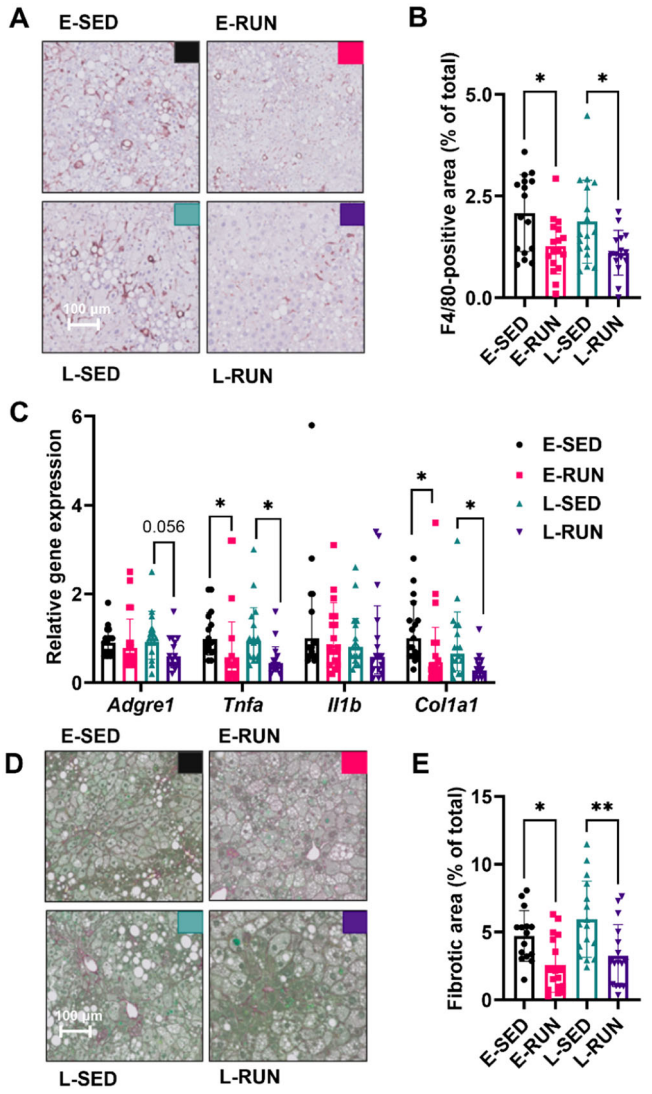

FIGURE 3 | Effect of early and late exercise training on liver inflammation and fibrogenesis. After 12 weeks of exercise training, (A, B) hepatic macrophages were visualized with an F4/80 staining. (C) The gene expression of pro-inflammatory and pro-fibrotic markers was measured and (D, E) liver fibrosis was quantified with a Sirius red/Fast green staining. Data are presented as geometric mean ± geometric SD in C, and as mean ± SEM ($n = 16–18$) in B and E, $^{*}p < 0.05$, $^{***}p < 0.01$ according to one-way ANOVA followed by Tukey's multiple comparisons test.

exercise training did not change the caloric output in the feces or intestinal permeability (Figure 5E,F).

As we saw an increase in SCFA producers in the gut, we quantified SCFA levels in the plasma of portal vein blood, which transports intestine-derived SCFAs to the liver, collected 19–28 h after the last training bout following 4 h fasting. While total SCFA levels (Figure 4H) that primarily reflected the abundance of acetate (Figure S5H), tended to be mildly increased in both exercising groups, there was no significant difference between the groups (Figure S5G, I–M). Curiously, despite a lack of significant difference in concentration between the exercise training groups, the total concentration of SCFAs in portal vein blood plasma negatively correlated with the steatosis MASLD score (Figure 4I). Additionally, the abundance of the *Lachnospiraceae* family, of which several members were increased only in L-RUN, correlated positively with the total SCFAs concentration in the portal vein plasma (Figure 4J). Taken together, these findings indicate that the reshaping of the gut microbiota with late exercise training may play a role in attenuating MASLD development.

3.5 | Fecal Microbiota Transplantation From Late-Trained Mice Tends to Decrease Liver Lipid Accumulation

To investigate if the gut microbiota changes observed with late exercise training are causal for the MASLD amelioration, we conducted an FMT experiment. Antibiotic-treated male APOE*3-Leiden.CETP mice received FMT from either sedentary (SED) mice or mice trained at ZT22 (RUN) for 8 weeks (Figure 5A). This experiment confirmed the beneficial effects of late exercise training on body weight and fat mass gain, plasma TG and TC levels and liver size in RUN mice (Figure 5B–G), as observed in experiment 1. Although not statistically significant, there was a clear trend for RUN FMT attenuating weight gain and plasma TG levels when compared with SED FMT (Figure 5D), while only exercise training itself decreased plasma TC in RUN (Figure 5E). Importantly, RUN FMT mice tended to have a lower liver weight (Figure 5F) and had a significantly lower liver/BW ratio similarly to RUN (Figure 5G). In line, both RUN and RUN FMT showed a reduced liver TG content compared to SED (Figure 5H). However, despite an approximate 15% decrease, there was no significant difference between RUN FMT and SED FMT, due to a large intra-group variability (Figure 5H). Histological analysis of the livers showed similar results, with a decreased MASLD score in RUN and a trend towards a reduced score in RUN FMT compared to the corresponding SED and SED-FMT groups (Figure 5I,J; Figure S6B). Similarly, a lipid staining of the liver showed a decrease in lipid accumulation in both RUN and RUN FMT compared to SED, but no significant difference compared to SED FMT (Figure 5K,L). Curiously, only RUN decreased inflammation in the liver based on the histological inflammation score and the gene expression levels of pro-inflammatory cytokines, with no inflammatory changes in RUN FMT (Figure S6C,D). These findings suggest that microbiota transplantation from trained into untrained mice confers some of the beneficial effects of late exercise training on the liver and whole body but with greater intra-group variability.

3.6 | FMT Leads to an Intestinal Passage Effect Rather Than Stable Engraftment of Transplanted Bacteria

While RUN FMT tended to improve overall metabolic and liver health, it is still unclear which bacteria or metabolites contributed to these changes. To investigate this further, we conducted 16S sequencing on representative cecum content samples of the donor and recipient mice. The antibiotics treatment before the FMT successfully depleted the gut microbiota of the recipient mice, clearly decreasing the Shannon diversity index from 3.2 ± 0.1 before the antibiotics treatment to 1.4 ± 0.3

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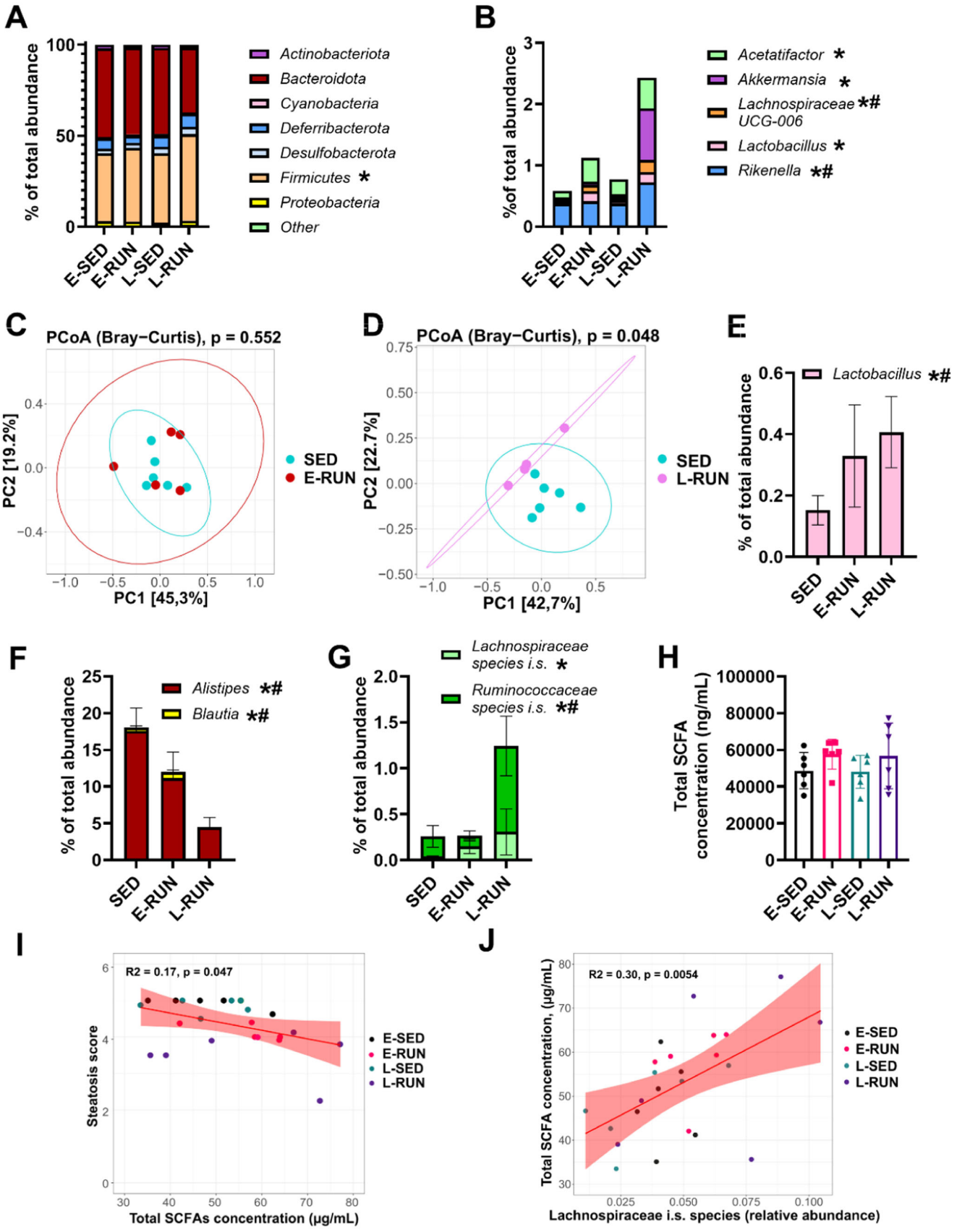

FIGURE 4 | Legend on next page.

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after antibiotics (Figure 5M). The analysis of the β-diversity also showed a change after the antibiotics treatment (Figure S6E). However, at the end of the study, only RUN showed a significantly different gut microbiota composition from both SED and RUN FMT, while there was no difference in β-diversity between SED FMT and RUN FMT (Figure 5N–Q). When dividing the RUN FMT mice into mice with high and low liver TG and MASLD score, mice with low TG and MASLD score (responders) had a similar microbiota composition to RUN mice, while mice with high liver TG and MASLD score had a composition more similar to SED (Figure 5R). This indicates that the success of the FMT engraftment varied and only a successful engraftment replicated the observed MASLD amelioration achieved by exercise training.

4 | Discussion

Exercise interventions are among the few available MASLD treatments, but little is known about optimizing these interventions in conjunction with our circadian rhythm [1, 9, 24, 25]. Here, we demonstrate for the first time in a MASLD mouse model that only late, but not early active phase exercise training effectively ameliorates MASLD development and that this effect is partially mediated via the gut-liver axis.

Clinically, weight loss is instrumental in the MASLD resolution, with already 5% weight loss leading to a significant improvement in steatosis [26]. While we observed a significant weight and fat mass loss with late exercise training that coincided with reduced steatosis, we curiously did not observe any differences in food intake or energy excretion between the groups, suggesting that altered energy utilization is the main mechanism underlying these positive effects of late training. This may be associated with the timing of food intake as the early-trained mice presumably ate the majority of calories after the training, while the late-trained animals ate the bulk before the training. However, as high-fat diet-fed mice have a disrupted circadian feeding rhythm with up to 30% of the food intake taking place during the light phase [27], it is unclear how much feeding timing contributed to the observed effects in this study. Nevertheless, we observed no differences between the groups in hepatic fatty acid metabolism-related gene expression or liver uptake of lipids or glucose. Since all these measurements were performed at rest 19–28 h after the last training bout, however, it is conceivable that late-trained mice achieved a greater use of energy during exercise but not at rest. While we did not see an increase in the protein abundance of the individual oxidative mitochondrial complexes or an increased uptake of the lipids or glucose by skeletal muscle at rest either, possibly due to samples being collected at ZT18, when muscle oxidative capacity is not at its peak, muscle oxidative capacity has been shown to be increased during training later in the day [28, 29]. This suggests greater energy utilization by muscle later in the active phase, especially paired with a possibly increased SCFA influx from the gut [30] which is known to increase muscle oxidative capacity further [31, 32]. Hence, we hypothesize that small acute effects during the daily exercise bouts accumulate into major effects after 12 weeks of training that lead to MASLD amelioration with late exercise training.

While only late exercise training reduced liver lipid accumulation, both early and late exercise training decreased markers of hepatic inflammation and fibrogenesis. This suggests that at a MASLD stage with pronounced steatosis and beginning fibrosis, inflammation and fibrogenesis are not regulated by the circadian aspect of exercise, possibly due to strong intra-hepatic disease drivers being no longer under circadian control. Additionally, while exercise-to-sedentary FMT tended to decrease liver lipid accumulation, it had no effect on liver inflammation, suggesting that the anti-inflammatory effect of exercise training is likely not directly mediated via the gut microbiota. This is in line with our observation that the gut microbiome was only changed in late-trained mice while early-trained mice also showed an amelioration of liver inflammation. Alternatively, this time-independent beneficial effect of exercise on liver inflammation may be explained by a modulation of hepatic cholesterol metabolism, as livers of both early and late-exercise-trained mice demonstrated decreased total cholesterol levels. This matches previous studies demonstrating that liver cholesterol is a determinant of liver inflammation [24, 33]. In line, the expression of the *Cyp7b1* gene of the alternative bile acid synthesis pathway was elevated in both exercise groups, potentially indicating increased cholesterol excretion in trained animals which in turn alleviates hepatic inflammation and pro-fibrotic processes.

An improved MASLD outcome is usually associated with beneficial changes in the gut microbiota composition in patients with MASLD [34] and establishing a healthy gut microbiota can even counteract weight re-grain in individuals following a lifestyle intervention [35]. In line, we here observe that only late, but not early exercise training modified the gut microbiota composition. A higher abundance of *Firmicutes* as seen in the late-trained animals has been correlated with higher VO_2 max, a marker of oxidative fitness [36–38]. Accordingly, this increase in *Firmicutes* seen with late training may also contribute to the higher exercise capacity humans exhibit later in the day compared to the morning [29]. *Akkermansia*, another genus enriched with late training here, has been linked to the amelioration of atherosclerosis, MASLD and type 2 diabetes, possibly via improved intestinal glucose control [39–42]. Also,

FIGURE 4 | Effect of early and late exercise training on gut microbiota composition. 16S sequencing of the cecum content was conducted after 12 weeks of training and the abundance of (A) bacterial phyla and (B) genera was quantified. Data are presented as mean ($n = 16$–18), *L-RUN vs L-SED, #L-RUN vs E-RUN, according to Wald test with FDR set to 0.05. In depth metagenomics sequencing of a subset of samples was conducted and (C, D) the β-diversity Bray-Curtis dissimilarity index calculated and groups compared with PERMANOVA. The abundance of genera (E) increased and (F) decreased in the L-RUN group was quantified, as well as (G) species increased in the L-RUN group. Data are presented as mean ± SEM ($n = 4$–6), *L-RUN vs L-SED, #L-RUN vs E-RUN, according to Wald test with FDR set to 0.05. Total SCFA concentration was measured in the portal vein blood plasma (H) and correlated to steatosis score (I) and the relative abundance of the identified increased bacterial species (J). Data are presented as mean ± SEM ($n = 5$–6) in (H) according to one-way ANOVA, and as a correlation plot according to Pearson correlation test (I–J).

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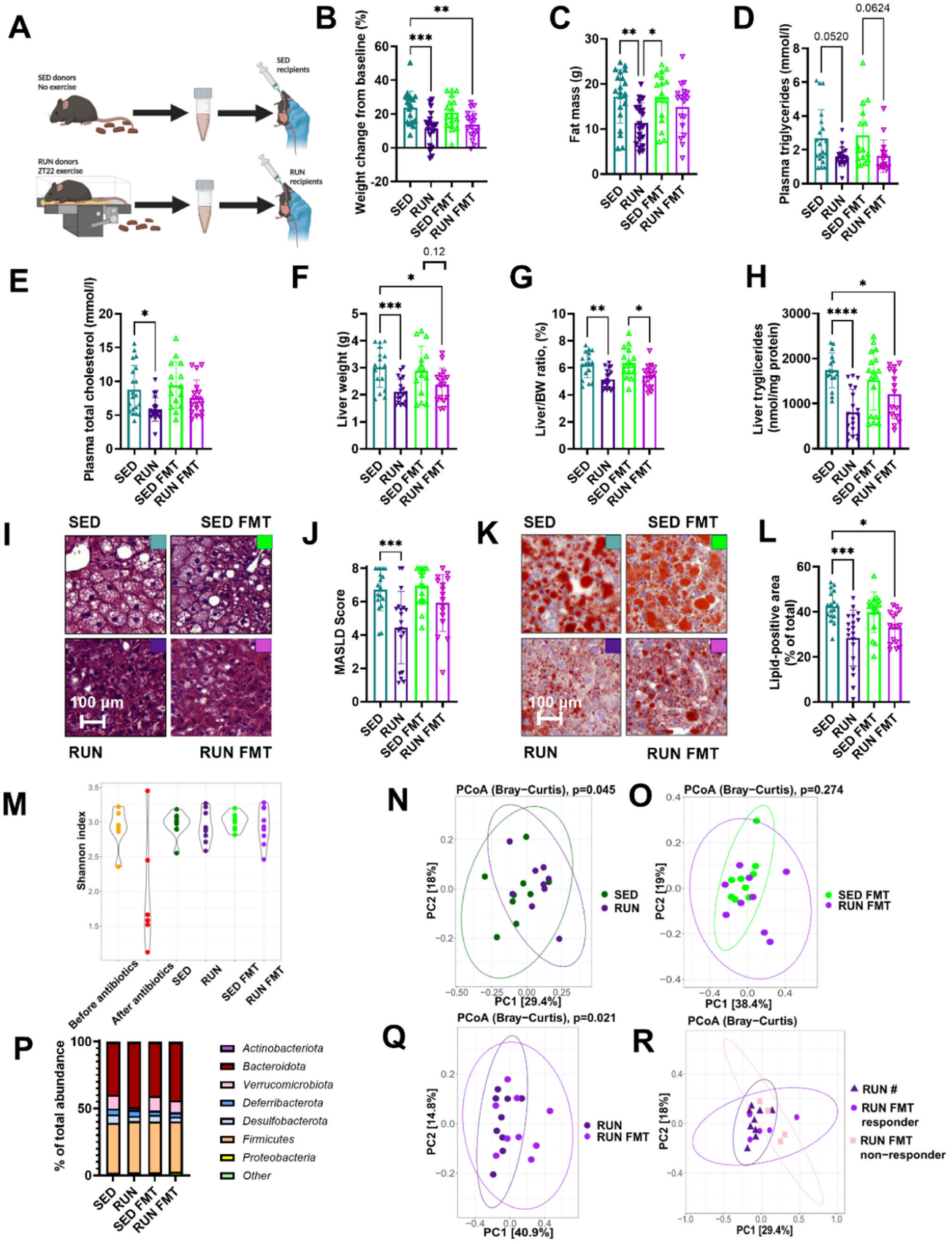

FIGURE 5 | Legend on next page.

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Lactobacillus has been associated with decreased liver fat, liver cholesterol and overall body fat loss [43–45], possibly via the competition for the available dietary fatty acids in the intestine. *Lachnospiraceae_UCG-006*, here enriched on genus level, has been associated with improved liver health as evident from decreased liver enzyme (ALT and AST) plasma levels in mice [46]. The observed late exercise-induced increase in unidentified members of the *Lachnospiraceae* and *Ruminococcaceae* families is especially interesting, as we have also observed their increase with late exercise training in a hyperlipidemic atherosclerosis-prone mouse model on a Western-type diet [15]. This indicates a robust effect of exercise training on these bacterial species in different mouse models on different diets. These broad families are known to ferment fiber into SCFAs, and SCFAs, especially butyrate, have health benefits, such as improved intestinal epithelial lining integrity, decreased inflammation in the gut and the liver, and increased muscle oxidative capacity [47–50]. In this study, possibly because portal vein blood was collected after 4 h of fasting 1 day after the last training bout, we did not see a clear difference in SCFAs levels between groups as they are quickly absorbed. On the other hand, the SCFA levels positively correlated with the abundance of SCFA-producing bacterial species and negatively correlated with MASLD scores. This may hint towards the involvement of SCFAs in MASLD amelioration in the current study and towards an effect that carries over into periods of rest from acute exercise training where it is likely more pronounced.

It is not clear why specifically late exercise training leads to an increase in the abundance of SCFA-producing bacteria. One explanation may be fluctuations in lactate production at different times of day by active skeletal muscle and the subsequent uptake of lactate into the gut during exercise as previously observed [51, 52]. In the gut, lactate can directly be used as an energy source and converted into SCFAs by gut bacteria [53–55]. While lactate production was not different during the morning and evening exercise in healthy volunteers [56], lactate levels were increased with late exercise training in mice [57] and the timing of exercise played a role in lactate production in patients with type 2 diabetes [58]. Additionally, the gut microbiota composition can itself oscillate, and in healthy conditions, the abundance of *Lactobacillus* [59] and *Lachnospiraceae* [60] and other SCFA producers is increased near ZT22, when the late exercise training took place. Assuming the rhythmicity of the gut microbiota oscillation is somewhat maintained under the high-fat diet conditions in this study, it is possible that during the late exercise training the greater abundance of SCFA producers leads to increased use of lactate or other metabolites as an energy source, which over time further promotes their proliferation and positive health changes. Acetate, the SCFAs produced in the highest quantities in the gut, is also instrumental for prolonged exercise endurance in mice [61], which corresponds to endurance being generally higher in the later part of the day [62].

To investigate the casual link between the altered microbiota and MASLD amelioration, we transplanted the microbiota of late-exercising into non-exercising mice. The findings from this experiment clearly support the role of the gut microbiota in later exercise-induced alleviation of liver steatosis. However, the MASLD amelioration was not as strong as in the exercising donor mice, for which there can be three explanations. Firstly, it is likely that the increased energy used by skeletal muscle during exercise substantially contributes to reduced liver lipid storage [63]. Secondly, variable engraftment of the donor microbiota may have led to a division of recipients into 'low responders' with unimproved liver health and "high responders" with ameliorated MASLD scores and reduced liver TG content. This distinction appeared to be dependent on the engraftment success of the FMT, which has been proposed to play an important role in the positive FMT effects [64]. Thirdly, while RUN donors were trained for twelve weeks, their FMT recipient counterparts only received the microbiota transplantation for 8 weeks in total. This was owed to the assumption that changes to the gut microbiota composition in response to an intervention like exercise training take up to 4 weeks to be fully established in the donor mice [65]. An extension of the FMT period past the termination of the donors was not done due to the improved survival of fecal microbes during an FMT from fresh rather than frozen feces [66, 67].

Overall, our findings show that only late, but not early exercise training is beneficial for the amelioration of steatosis in MASLD and that the associated changes to the composition of the gut microbiota likely play a causal role in this effect. Along with the improvement of liver steatosis with late exercise training, we observe an increase in beneficial bacterial taxa that are known to produce SCFAs, to compete for the use of dietary lipids, and generally improve health outcomes. This positive effect of late exercise training on the whole body and the liver was partially transferrable via a FMT, supporting the importance of a healthy gut-liver axis in the prevention and treatment of MASLD. Nonetheless, both early and late exercise trainings decreased inflammation in the liver. This suggests that any form of exercise is still preferable to sedentary behavior. While a multitude of additional factors such as chronotype, sleep quality and meal timing are to be considered for the optimal design of

FIGURE 5 | The effect of fecal microbiota transplantation from late-trained into untrained mice on MASLD amelioration. (A) Feces from sedentary (SED) and late-trained (RUN) mice were transplanted to corresponding sedentary groups (SED FMT and RUN FMT) for eight weeks. At the end of the experiment, (B) weight change, (C) fat mass, (D) plasma triglycerides and (E) total cholesterol were quantified. To investigate liver changes, (F) liver weight, (G) liver-to-body weight ratio and (H) liver triglycerides were measured, (I) H&E staining was performed to calculate (J) the MASLD score and (K) ORO staining was performed to measure (L) the amount of lipids in the liver sections. To investigate microbiota composition changes, 16S sequencing was performed, and (M) α-diversity, (N, O, Q) β-diversity between groups and (R) β-diversity difference between RUN, RUN FMT responders and RUN FMT non-responders was quantified. The phylum abundance (P) in the groups was measured. (B–L) are presented as mean ± SEM ($n = 17–20$), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ according to one-way ANOVA followed by Tukey's multiple comparisons test. Differences between the groups in (N–O, Q–R) were calculated with PERMANOVA with Bonferroni correction, with test results shown on top of the panel for N, O, Q, and $^{\#}$showing comparison between RUN and RUN FMT non-responders, where $^{\#}$stands for $p < 0.05$, while differences in (P) were calculated according to Wald test with FDR set to 0.05. Unless specified otherwise, only significant comparisons are shown.

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clinical lifestyle interventions, we here present the first proof that late exercise training may be a better treatment strategy than early exercise for the amelioration of MASLD.

Author Contributions

Artemiy Kovynev, Zhixiong Ying, Patrick C. N. Rensen and Milena Schönke designed the experiments; Artemiy Kovynev, Zhixiong Ying, Sen Zhang, Emanuele Olgiati, Joost M. Lambooij, Clara Visentin and Milena Schönke collected and analyzed the data; Artemiy Kovynev, Zhixiong Ying and Milena Schönke drafted the manuscript; Bruno Guigas, Quinten R. Ducarmon and Patrick C. N. Rensen critically revised the manuscript.

Acknowledgements

The authors thank Milaine V. Hovingh, Jan Freark de Boer, Christiana Costa, Amanda Pronk, Salwa Afkir, Trea Streefland, Reshma Lalai, Chris van der Bent, Frank Otto, and Hetty Sips for their excellent technical assistance. This work was supported by the Novo Nordisk Foundation (grant NNF18OC0032394 to M.S.), The Netherlands Cardiovascular Research Initiative CVON-GENIUS-2 (grant to P.C.N.R.) and the Chinese Scholarship Council (grants to Z.Y. and S.Z.). A.K. is supported by a PhD grant from Leiden University Medical Center (to M.S.).

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data Availability Statement

The data that support the findings of this study are openly available in ENA at https://www.ebi.ac.uk/ena/browser/home, reference number PRJEB78442. 16S and metagenomics data are available from the ENA database (ascension number PRJEB78442).

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