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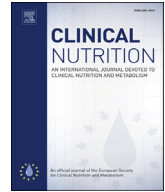
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Randomized Control Trials

Effect of a 24-week supervised concurrent exercise intervention on fecal microbiota diversity and composition in young sedentary adults: The ACTIBATE randomized controlled trial

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SUMMARY

Background: Numerous physiological responses to exercise are observed in humans, yet the effects of long-term exercise and varying intensities on the diversity and composition of human fecal microbiota remain unclear. We investigated the effect of a 24-week supervised concurrent exercise intervention, at moderate and vigorous intensities, on fecal microbiota diversity and composition in young adults.

Methods: This ancillary study was based on data from the ACTIBATE randomized controlled trial (ClinicalTrials.gov ID: NCT02365129), and included adults (aged 18–25 years, 70 % female) that were randomized to (i) a control group (CON: no exercise, n = 20), (ii) a moderate-intensity exercise group (MOD-EX, n = 21), and (iii) a vigorous-intensity exercise group (VIG-EX, n = 20). Fecal samples were collected before and after the 24-week exercise intervention, and the diversity and composition of the fecal

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Resistance exercise
Succinate

microbiota were analyzed by 16S rRNA sequencing. Inferential functional profiling of the fecal microbiota was performed and correlations between microbial changes and cardiometabolic outcomes were assessed.

Results: Exercise did not modify beta or alpha diversities regardless of the intensity (all $P \geq 0.062$). The relative abundance of the *Erysipelotrichaceae* family (*Bacillota* phylum) ($-0.3 \pm 1.2\%$; $P = 0.031$) was however reduced in the VIG-EX group. *Coprococcus* was the only genus showed a significant difference between MOD-EX and VIG-EX after the intervention, with its relative abundance increasing in MOD-EX ($+0.4 \pm 0.6\%$; $P = 0.005$). None of these changes were related to the exercise-induced cardiometabolic benefits (all $P \geq 0.05$).

Conclusions: In young adults, a 24-week supervised concurrent exercise program, at moderate and vigorous intensities, resulted in minor changes in fecal microbiota composition, while neither alpha nor beta diversities were affected.

Clinical trial registration: ClinicalTrials.gov ID: NCT02365129.

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1. Introduction

The gastrointestinal tract is colonized by microorganisms such as Eukarya, Archaea, fungi, and mainly bacteria [1], the so-called gut microbiota. There are five dominant phyla in the human gut, of which *Bacillota* and *Bacteroidota* are more abundant ($>75\%$) compared to *Pseudomonadota*, *Verrucomicrobiota*, and *Actinomycetota* ($<25\%$) [2]. Collectively, these microorganisms compose a gut ecosystem that could be in a balanced state known as eubiosis or in an imbalanced state referred as dysbiosis [3]. Recent studies have shown that these gut bacteria seem to regulate host physiology, metabolism, nutrition, and immune system development, being of great interest to the scientific community [4–6].

Exercise is known to modify whole-body metabolic activity [7], and it is considered a non-pharmacological therapy to prevent and combat cardiometabolic diseases [8,9]. However, the molecular mechanisms driving exercise's beneficial effects in humans are poorly understood. Recent studies suggest that exercise improves cardiometabolic health by modifying the gut microbiota composition [10–15]. In fact, it has been demonstrated that exercise training, specifically running, restores eubiosis in the gut of metabolically compromised mice [16–18]. Additionally, we [11] and others [12–15] have observed a positive correlation between exercise and increased fecal microbiota diversity, along with a higher relative abundance of *Bacillota* and *Bacteroidota* in humans [19–26]. Exercise intervention studies have shown that 6–12 weeks of endurance training modifies the fecal microbiota composition in normal-weight individuals as well as in adults with overweight and obesity [27–29]. The combination of moderate to vigorous aerobic exercise with muscle-strengthening activities is seemingly the most effective approach for sustaining or enhancing physical fitness and overall health [30]. In this context, concurrent training interventions, integrating both aerobic and resistance training, emerge as a potentially optimal approach to maximize the benefits of exercise. Little is known about the impact of concurrent exercise training on fecal microbiota diversity and composition in humans. Moreover, considering the emphasis on exercise intensity in current physical activity recommendations [30], there is a compelling need for a comprehensive investigation to determine whether higher exercise intensity confers additional benefits compared to moderate intensity.

Therefore, this randomized controlled trial investigated the effect of a 24-week supervised concurrent exercise intervention, incorporating both moderate and vigorous intensities, on fecal microbiota diversity and composition in young adults.

2. Material and methods

2.1. Participants

A total of 145 adults aged between 18 and 25 years old participated in the ACTIBATE study [31,32] (Fig. 1), an exercise-based randomized controlled trial (ClinicalTrials.gov ID: NCT02365129) that aimed to investigate whether a 24-week exercise intervention was able to activate and recruit human brown adipose tissue (BAT). The criteria for participation in the study included: (i) a sedentary lifestyle, defined as engaging in physical activity for less than 20 min on fewer than 3 days per week, and (ii) maintaining a stable body weight, with less than a 3 kg change, over the preceding 3 months. The exclusion criteria were: (i) being a smoker, (ii) having any illness that could restrict the ability to participate, (iii) taking medication impacting the cardiovascular system or gut microbiota, like antibiotics, or (iv) being pregnant. The participants were recruited from the province of Granada (Spain) through social networks, local media, and posters. The study protocol and the written informed consent were performed following the last revised Declaration of Helsinki (2013) and were approved by the Ethics Committee on Human Research of the University of Granada (n°.924) and Servicio Andaluz de Salud (Centro de Granada, CEI-Granada). All participants signed informed consent.

2.2. Study design

The current manuscript is an ancillary study from the single-center ACTIBATE study [32], a randomized controlled trial whose detailed design is described elsewhere [31] and which followed the CONSORT guidelines (Table S1). After the baseline (before the exercise intervention) examination (Table 1), the participants were randomly assigned into three different groups using a computer-generated simple randomization [33]: (i) control (CON), (ii) moderate-intensity exercise (MOD-EX), and (iii) vigorous-intensity exercise (VIG-EX). All recruited participants were instructed to keep their daily routine, physical activity, and/or dietary patterns. All measurements were conducted before and 24-weeks after the supervised training program.

2.3. Supervised exercise training programs

A detailed description of the supervised concurrent exercise training program can be found elsewhere [31]. Participants engaged in the program 3–4 times weekly, comprising 80 min of resistance exercise and 150 min of endurance training, in line with

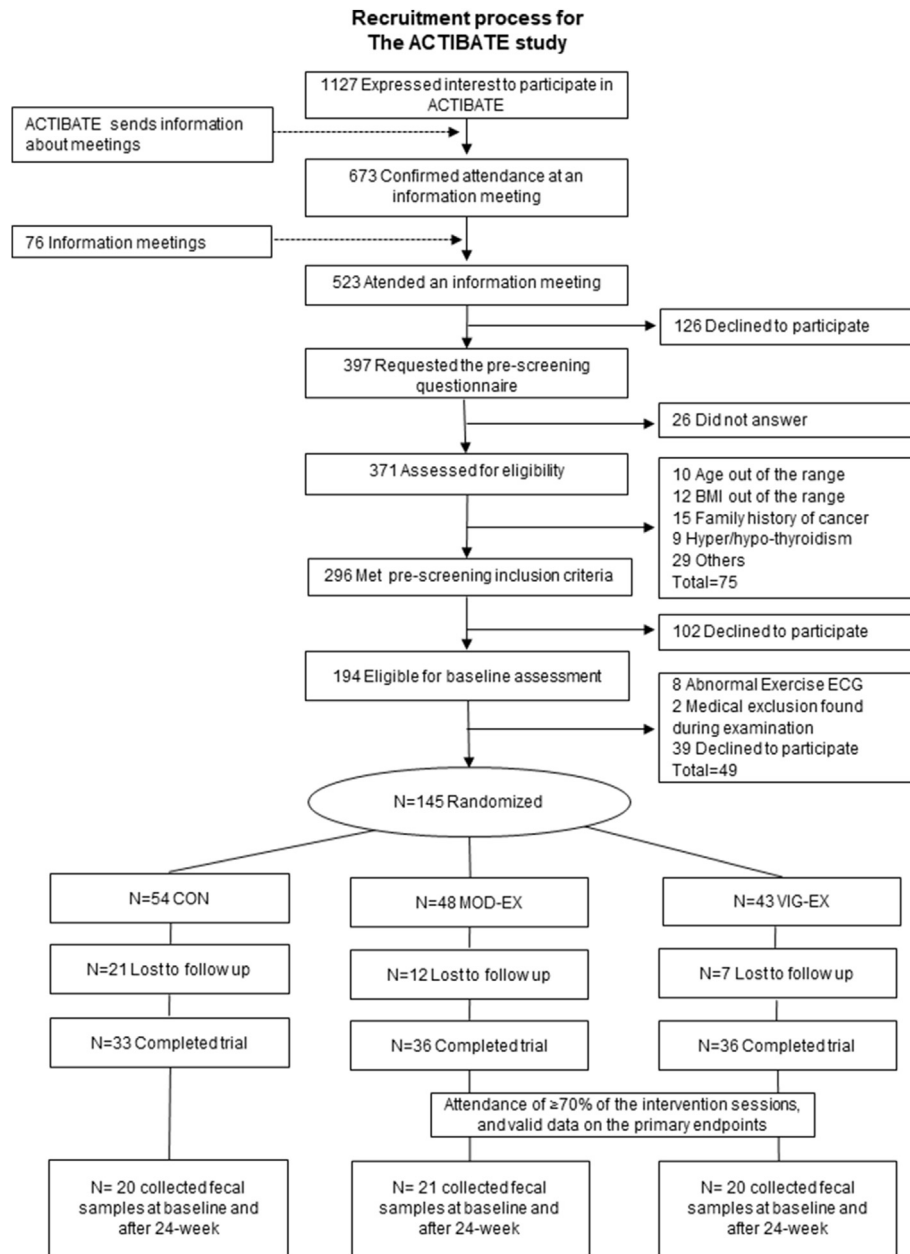


Fig. 1. Flow-chart diagram of the study participants. Abbreviations: BMI: body mass index; CON: control group; ECG: electrocardiogram; MOD-EX: moderate intensity exercise group; VIG-EX: vigorous intensity exercise group.

the WHO guidelines. The program was tailored to the individual physical fitness levels of each participant, ensuring both resistance and endurance exercises were appropriately personalized. MOD-EX and VIG-EX performed the resistance training at 50 % of the one repetition maximum (RM) and 70 % RM, respectively. The intensity of resistance exercises was individually adjusted monthly. In the MOD-EX, endurance training was performed at 60 % of the heart rate reserve (HRres), whereas the VIG-EX performed 75 min/week at 60 % HRres and 75 min/week at 80 % HRres.

Exercise training sessions were performed in groups of 10–12 participants. They attended the research center at the same time of the day during the 24-week program: 8.30 a.m.–10.30 a.m., 4 p.m.–6 p.m., 6 p.m.–8 p.m., and 8 p.m.–10 p.m. An electronic attendance sheet was used to register attendance, whereas heart rate monitors (Polar Electro Oy, Kempele, Finland) were used to quantify the adherence to the intensity prescribed for endurance

training. To ensure participants' attendance and adherence to the training program, they were allowed to do the workout outside the research center if they were unable to attend.

2.4. Fecal microbiota analysis

2.4.1. Fecal DNA extraction

Fecal samples (50–60 g) were obtained before and immediately after the intervention (48–72 h after the last exercise session) in a subsample of 61 individuals ($n = 20$ in CON, $n = 21$ in MOD-EX, and $n = 20$ in VIG-EX). These samples were collected from all participants, regardless of BMI. Fecal samples were collected and placed in sterile 60 mL plastic containers. These were stored in the home freezer before being transported to our laboratory in a portable cooler with an ice plate. Upon arrival, the fecal samples were stored at -80°C until DNA extraction. We used the QIAamp DNA Stool

Table 1
Baseline characteristics of the study participants.

	CON (n = 20)	MOD-EX (n = 21)	VIG-EX (n = 20)
	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD
Demographics			
Age (years old)	22.2 $\pm$ 2.2	21.9 $\pm$ 2.3	21.5 $\pm$ 2.4
Sex			
Male (n/%)	7 $\pm$ 35	5 $\pm$ 24	6 $\pm$ 30
Female (n/%)	13 $\pm$ 65	16 $\pm$ 76	14 $\pm$ 70
Body composition			
BMI (kg/m ²)	24.1 $\pm$ 4.3	26.0 $\pm$ 5.3	24.8 $\pm$ 4.6
Overweight (n, %)	4 $\pm$ 20	4 $\pm$ 19	9 $\pm$ 45
Obesity (n, %)	2 $\pm$ 10	6 $\pm$ 28.6	2 $\pm$ 10
Lean mass (kg)	41.5 $\pm$ 10.1	42.4 $\pm$ 8.7	41.7 $\pm$ 8.2
Fat mass (kg)	23.7 $\pm$ 8.4	27.9 $\pm$ 10.8	25.8 $\pm$ 8.1
Fat mass (%)	34.9 $\pm$ 8.5	38.0 $\pm$ 7.7	36.5 $\pm$ 7.1
VAT mass (g)	321.8 $\pm$ 178.4	380.4 $\pm$ 220.2	344.1 $\pm$ 178.7
Cardiometabolic profile			
Glucose (mg/dL)	88.4 $\pm$ 7.3	88.7 $\pm$ 6.6	86.4 $\pm$ 5.2
Insulin (U/mL)	8.7 $\pm$ 6.8	8.6 $\pm$ 3.7	8.7 $\pm$ 4.3
HOMA-IR	2.0 $\pm$ 1.8	1.9 $\pm$ 0.9	1.9 $\pm$ 1.0
TC (mg/dL)	156.6 $\pm$ 35.9	165.3 $\pm$ 32.3	173.6 $\pm$ 33.5
HDL-C (mg/dL)	51.6 $\pm$ 10.1	51.7 $\pm$ 14.0	52.4 $\pm$ 14.4
LDL-C (mg/dL)	89.3 $\pm$ 31.2	98.0 $\pm$ 28.2	100.3 $\pm$ 21.3
TG (mg/dL)	78.6 $\pm$ 53.1	87.9 $\pm$ 70.8	102.1 $\pm$ 49.3
CRP (mg/L)	2.1 $\pm$ 2.1	3.5 $\pm$ 4.4	2.6 $\pm$ 2.9
Succinate (μ M) ^a	63.7 $\pm$ 15.2	65.2 $\pm$ 26.2	76.6 $\pm$ 25.6
Dietary intake			
Energy (kcal/day)	1905.2 $\pm$ 381.2	1911.8 $\pm$ 470.7	1919.2 $\pm$ 465.7
Fat (g/day)	84.6 $\pm$ 21.1	85.4 $\pm$ 24.9	87.4 $\pm$ 21.9
Protein (g/day)	76.7 $\pm$ 21.0	77.0 $\pm$ 22.8	80.7 $\pm$ 18.4
Carbohydrate (g/day)	205.2 $\pm$ 63.5	202.9 $\pm$ 63.1	198.8 $\pm$ 65.0
Fiber (g/day)	17.2 $\pm$ 6.8	16.8 $\pm$ 5.6	15.4 $\pm$ 4.1

Data are presented as mean and standard deviation, except for sex, overweight and obesity (presented as n and percentage). No significant differences were observed in body composition, cardiometabolic profile, and dietary intake variables at baseline across groups (all $P \geq 0.8$, data not shown). **Abbreviations:** BMI: Body mass index, CON: Control group, CRP: C-reactive protein, HDL-C: high-density lipoprotein cholesterol, HOMA-IR: homeostatic model assessment-insulin resistance index, LDL-C: low-density lipoprotein cholesterol, MOD-EX: moderate intensity exercise group, TC: Total cholesterol, TG: triacylglycerol, VAT: Visceral adipose tissue, VIG-EX: vigorous intensity exercise group.

^a For plasma levels of succinate, 5 participants were missing (CON n = 19; MOD-EX n = 18; VIG-EX n = 19).

Mini Kit (QIAGEN, Barcelona, Spain) to extract and purify DNA according to the manufacturer's instructions. Finally, we used the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) to measure the DNA concentrations in the samples.

2.4.2. Sequencing

Extracted DNA was amplified by PCR targeting the V3 and V4 hypervariable regions of the bacterial 16S rRNA gene with the following primer pairs: forward Primer (CCTACGGGNGGCWGCAG) and reverse Primer (GACTACHVGGGTATCTAATCC). All PCRs were carried out in 25 μ L final reaction volume, including: 12.5 μ L of the 2X KAPA HiFi Hotstart prepared mixture (KAPA Biosystems, Woburn, MA, USA), 5 μ L of each forward and reverse primers (1 μ M), and 2.5 μ L of extracted DNA (10 ng). All PCRs utilized the following program: (i) denaturation at 95 °C for 3 min, (ii) 8 denaturation cycles at 95 °C for 30 s, (iii) annealing at 55 °C for 30 s, (iv) elongation at 72 °C for 30 s, (v) a final extension at 72 °C for 5 min. Next, we applied AMPure XP microspheres (Beckman Coulter, Indianapolis, IN, USA) to purify the 16S V3 and V4 amplicon away from XT primers and primer-dimer species. Then, we used the Nextera XT index kit (Illumina, San Diego, CA, USA) to tag DNA with sequencing adapters. The pooled PCR products were purified

with AMPure XP balls (Beckman Coulter, Indianapolis, IN, USA) before quantification. Lastly, we use the Illumina MiSeq paired-end sequencing system (2 $\times$ 300nt) (Illumina, San Diego, CA, USA) to sequence amplicons.

2.4.3. Bioinformatics: determination of fecal microbiota diversity, composition, and function

The *dada 2* [34] package version 1.10.1 in R software [35] was used for analyzing the raw sequences (FastQ files). Following a 10,000-read cut-off, all samples were considered valid for further analysis. Samples were standardized to an equal number of 30,982 reads with the *phyloseq* [36] package in R software, obtaining a total of 19,373 phylotypes. Phylotypes were assigned to their specific taxonomic affiliation (from phylum to genus) based on the naive Bayesian classification with a pseudo-bootstrap threshold of 80 % [36] using the *Classifier* function in Ribosomal Data Project (RDP) [37]. To determine species taxonomies, we used the *Seq-match* function in RDP according to criteria published previously [38].

2.4.4. Diversities

Beta diversity measures the number of species shared among the microbial community by the participants [39]. Alpha diversity is a measurement of the number of different phylotypes and the relative abundance of these phylotypes within the same participant [39]. We applied four proxies in this study for calculating alpha diversity: i) *species richness*, which is the count of different phylotypes observed in the bacterial community [40]; ii) *evenness index*, which is the equitability of phylotype frequencies in the bacterial community [39]; iii) *Shannon index*, which is the number and equitability of the phylotypes observed in the bacterial community [40], and iv) *inverse Simpson index* that is calculated by the classical Simpson index, and it is referred as the richness in a community with uniform evenness [41].

2.4.5. Composition

We obtained 16 different phyla and 240 genera. For the main analysis (see below for extended information), we used relative abundances as of the reads percent of each phylotype in relation to the total number of reads. We performed the analyses when the average of the relative sequence abundance was higher than 1 %.

2.4.6. Function

We annotated microbial genes according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology (KOs). The metabolic and functional contribution was inferred from 16S rRNA gene data using the Tax4fun [42] program built-in Galaxy [43] private instance of the DengLab [44] (<http://mem.rcees.ac.cn:8080>). For the present study, we included estimations of the amino acid, carbohydrate, energy, lipid, and nucleotide metabolisms (54 pathways in total) and functional succinate pathways (16 pathways).

2.5. Body composition

We measured the participants' weight and height barefoot, without shoes, and wearing light clothing, using a SECA scale and stadiometer (model 799; Electronic Column Scale, Hamburg, Germany). The body mass index (BMI) was calculated as weight/height² (kg/m²). Body composition (fat, lean, and visceral adipose tissue masses) was measured using a dual-energy X-ray absorptiometry scanner (Hologic Discovery Wi, Marlborough, MA). The fat

mass percentage was determined as the body fat mass divided by the total body mass and multiplied by 100.

2.6. Cardiometabolic profile

Blood samples were collected after 10-h overnight fasting, between 08:00 and 09:00 a.m.. Serum (obtained with Vacutainer® SST™ II Advance tubes) and plasma (obtained with Vacutainer® Hemogard™ tubes) were centrifuged directly in the laboratory (10,000 rpm, 10 min, 4 °C temperature). The samples were then stored in a –80 °C freezer for future analysis. Serum glucose, total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), and triacylglycerol concentrations were assessed following standard methods using an AU5832 biochemical analyzer (Beckman Coulter Inc., Brea CA, USA). Low-density lipoprotein-cholesterol (LDL-C) was estimated with the Friedewald formula [45]: $[TC - HDL-C - (triacylglycerols/5)]$, in mg/dL [45]. Insulin was measured using the Access Ultrasensitive Insulin Chemiluminescent Immunoassay Kit (Beckman Coulter Inc., Brea CA, USA). The homeostatic model assessment (HOMA) index was calculated as $[\text{insulin } (\mu\text{U/mL}) \times \text{glucose (mmol/L)}] / 22.5$ [46]. C-reactive protein (CRP) was measured by immunoturbidimetric assay, employing the same AU5832 automated analyzer. Finally, plasma succinate levels were measured using the EnzyChrom™ Succinate Assay Kit (BioAssay Systems, Hayward, CA). The assay sensitivity was 12 μM , and the intra- and inter-assay coefficients of variance were <3.50 % and 6.95 %, respectively, and the accuracy ranged from 1 to 11.5 % error [47,48].

2.7. Dietary intake

The EvalFINUT® software was used to assess the dietary intake (energy, macronutrient, and fiber intake) from three 24-h dietary recalls, as we extensively described elsewhere [49]. Qualified and trained dietitians undertook the 24-h food recalls face-to-face on 3 separate days (2 weekdays and one weekend day). In the software, all data from interviews were collected independently by two dietitians.

2.8. Statistical analysis

This study was ancillary to a parent study aimed at determining the effects of a 24-week supervised exercise intervention on BAT, which was originally powered to detect changes in BAT volume and activity. Therefore, no specific power calculation was performed for the current study. The descriptive parameters are reported as mean and standard deviation unless otherwise stated. Only participants who attended at least 70 % of the prescribed training sessions were included in the main analysis. All analyses were conducted for men and women together as no sex*exercise interaction on fecal microbiota analyses was detected (all $P \geq 0.05$; data not shown). Normality of data distribution was tested using the Kolmogorov–Smirnov test with Lilliefors correction. Alpha diversity indices followed a normal distribution and were analyzed using parametric methods. In contrast, phenotypic traits and microbiota composition did not meet the assumptions of normality and were therefore analyzed using non-parametric methods. Kruskal–Wallis analysis was applied to baseline phenotypic traits, followed by post-hoc adjustments using the Benjamini–Hochberg false discovery rate (FDR). Baseline microbiota composition was assessed using the “DESeq2” package in R software, identifying differentially abundant taxa between groups with P-values adjusted for multiple comparisons using the FDR method. Permutational multivariate analysis of variance (PERMANOVA) with 9999 random permutations based on Bray–Curtis dissimilarity was used

to investigate differences in beta diversity between groups in Paleontological Statistics Software Package 3.0 (Past3) [50]. With the *vegan* package in R software, we visualized beta diversity relationships at phylum and genus levels using Principal coordinate analysis (PCoA) in two-dimensional scatter plots. Generalized linear mixed models (GLMM) with gamma distribution and log link were used to assess differences in phenotypic variables as well as functional pathways associated with the microbiota over time between study groups. This approach was chosen due to the non-normal nature of the data and to adequately handle repeated measures within individuals by including a random effect specific to each participant. For variables related to microbial abundance, GLMMs with a negative binomial distribution and a zero inflation model were used to account for the overdispersion and high frequency of zeros in the data. In both cases, the models included the main effects of group, time, and their interaction (group $\times$ time) as predictors. Coefficients of the fixed effects were extracted for each variable and p-values were adjusted using the FDR method. All analyses were performed with the “glmmTMB” and “lme4” packages in R software. To evaluate dynamic changes in microbiota composition in response to the exercise intervention, we conducted both within-group and between-group analyses.

To study the effect of exercise on fecal microbiota diversity, we conducted analyses of covariance (ANCOVA) with baseline values included as covariates. Additionally, Bonferroni post-hoc adjustments were utilized for multiple comparisons to identify differences among the groups. A delta (Δ ; post minus baseline values) was calculated for each outcome. For partial correlation analyses, we used the “psych” and “corrplot” packages in R software to compute Spearman partial correlation coefficients (ρ) between changes in microbiota outcomes and changes in body composition, cardiometabolic profile, and dietary intake outcomes, adjusting for both sex and age.

The significance level was set at $P < 0.05$. We used the Statistical Package for Social Sciences (SPSS, v.22.0, IBM SPSS Statistics, IBM Corporation) and R software to perform the statistical analysis. Graphical plots were generated with GraphPad Prism v.7 (GraphPad Software, San Diego, CA, USA) and the “gplot” package in R software.

3. Results

Among the 145 participants enrolled in the ACTIBATE study, 61 had fecal microbiota measurements and successfully finished the exercise training program, and were therefore included in the main analyses (see flowchart in Fig. 1). At baseline, beta diversity was similar across groups at phylum and genus taxonomic levels (all $P \geq 0.205$; Fig. 2A). Similarly, there were no differences in alpha diversity parameters (i.e., *species richness*, *evenness index*, *Shannon index*, or *inverse Simpson index*; all $P \geq 0.053$; Fig. 2B), or fecal microbiota composition (all $P \geq 0.083$; Fig. 2C, Fig. S1).

3.1. 24-weeks of supervised concurrent exercise intervention does not modify fecal microbiota diversity, whereas it slightly modifies fecal microbiota composition

The exercise interventions did not alter the fecal microbiota beta (all $P \geq 0.086$, Table 2) and alpha diversities (all $P \geq 0.062$, Fig. 3). However, the relative abundance of the *Erysipelotrichia* class, *Erysipelotrichales* order and *Erysipelotrichaceae* family (*Bacillota* phylum) was decreased in the VIG-EX group in comparison to CON [*Erysipelotrichaceae* family $\Delta \text{ CON} = 0.4 \pm 1.4 \%$; $\Delta \text{ MOD-EX} = 0.0 \pm 0.4 \%$; $\Delta \text{ VIG-EX} = -0.4 \pm 1.2 \%$; $P = 0.048$, Fig. 4 and Table S2].

In the analysis of the dynamic changes within each group, a significant change was observed only in the MOD-EX group, where

the abundance of the genus *Coprococcus* increased after the intervention. Furthermore, *Coprococcus* genus was the only bacterium that showed statistically significant differences after the intervention when comparing the MOD-EX and VIG-EX groups [Δ MOD-EX = 0.4 ± 0.6 %; Δ VIG-EX = -0.0 ± 0.5 %; $P = 0.005$, Table S2].

No significant changes in fecal functional pathways were observed between groups after the intervention. However, there was a trend toward downregulation in the VIG-EX group for the glycerolipid pathway (ko00561; nominal $P = 0.011$, adjusted $P = 0.065$) and the glyoxylate and dicarboxylate pathways (ko00630; nominal $P = 0.018$, adjusted $P = 0.072$), although these did not reach statistical significance after FDR correction (data not shown).

3.2. The exercise-induced changes in fecal microbiota composition are independent of exercise-induced changes in body fat, cardiometabolic profile, and dietary intake

MOD-EX and VIG-EX reduced total fat mass (kg) [Δ CON = 0.0 ± 3.9 kg; Δ MOD-EX = -1.6 ± 2.3 kg ($P = 0.05$); Δ VIG-EX = -3.3 ± 4.0 kg ($P = 0.005$), Table S3]. Moreover, VIG-EX reduced fat mass percentage, [Δ CON = 0.5 ± 5 %; Δ MOD-EX = -2.8 ± 3.0 %; Δ VIG-EX = -3.9 ± 3.4 %; $P = 0.034$, Table S3]. MOD-EX reduced LDL [Δ CON = 11.1 ± 14.0 mg/dL; Δ MOD-EX = -4.7 ± 18.5 mg/dL; Δ VIG-EX = 1.4 ± 19.2 mg/dL; $P = 0.034$,

Table 2
Effect of a 24-week supervised concurrent exercise intervention on beta diversity.

Taxonomic levels	Group of study (CON, MOD-EX and VIG-EX)	
	Pseudo-F	P
Phylum	1.16 E + 27	0.086
Class	72.360	0.518
Order	8.24 E + 25	0.120
Family	-14.270	0.797
Genus	-18.740	0.979

PERMANOVA using 9999 permutations for significance testing ($P < 0.05$). Abbreviations: CON: Control group, MOD-EX: moderate intensity exercise group, Pseudo-F: statistic, larger number indicates greater separation [70] across groups of study, VIG-EX: vigorous intensity exercise group.

Table S3] whereas increased energy intake [Δ CON = -94.6 ± 546.5 kcal/day; Δ MOD-EX = 295.6 ± 571.4 kcal/day; Δ VIG-EX = 218.7 ± 564.5 kcal/day; $P = 0.019$, Table S3], and dietary fiber intake [Δ CON = -2.9 ± 6.6 g/day; Δ MOD-EX = 3.7 ± 7.1 g/day; Δ VIG-EX = 1.4 ± 7.2 g/day; $P = 0.023$, Table S3] compared to the CON. However, no significant correlations were found between changes in any of these parameters and the changes in the relative abundance of the aforementioned bacteria (i.e., *Erysipelotrichaceae* family) (all $P > 0.05$ Fig. S2). An exploratory analysis was conducted to identify potential correlations between changes in bacterial abundance and phenotypic traits, with the goal

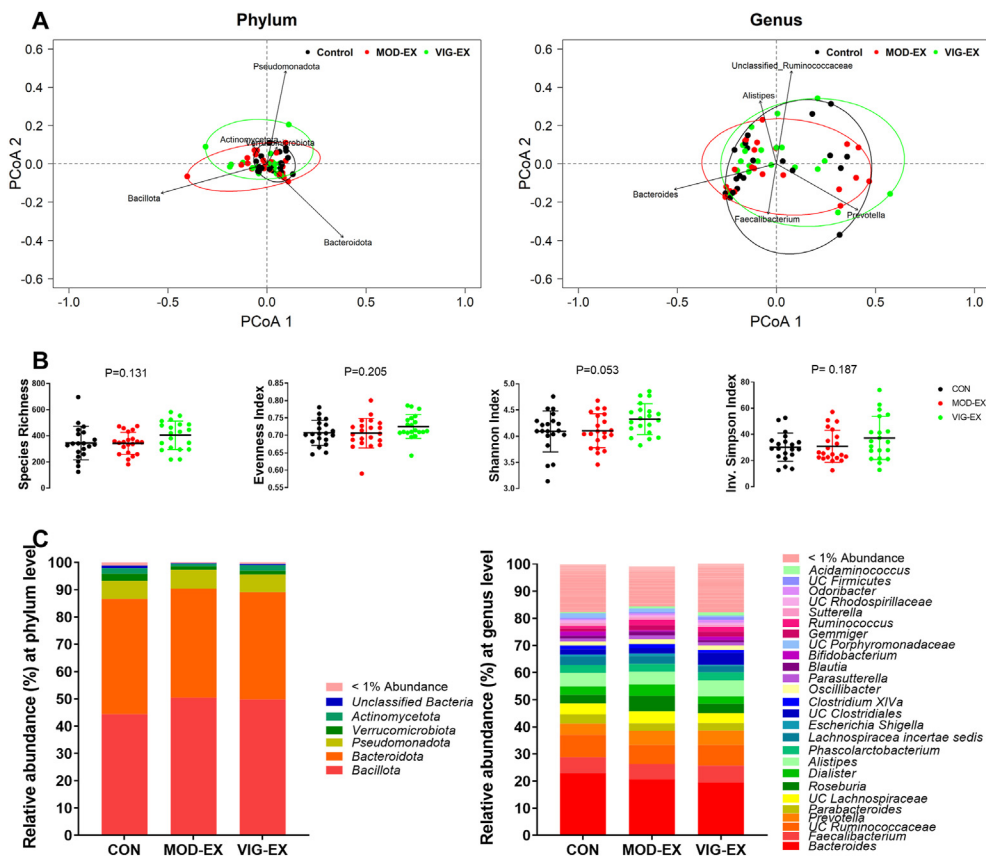


Fig. 2. Descriptive fecal microbiota diversity and composition parameters characteristics at baseline. Panel A shows differences in the fecal microbiota community structure at phylum and genus taxonomic levels visualized by principal coordinate ordination analyses (PCoA) using Bray–Curtis dissimilarity; ellipses represent the 95 % confidence interval for every single group. Genus PCoA only shows the five genera of higher abundance. Panel B shows different indexes of fecal microbiota alpha diversity (species richness, evenness index, Shannon index, and inverse Simpson index). Panel C indicates the relative abundance of the fecal microbiota composition at the phylum and genus taxonomic level. The stacked bar represents the percentage of relative abundance. All the results are depicted by CON ($n = 20$), MOD-EX ($n = 21$), and VIG-EX ($n = 20$). P values were obtained from a one-way analysis of variance. Bonferroni was used to adjust multiple comparisons across groups. Abbreviations: CON: control group; MOD-EX: moderate intensity exercise group; UC: unclassified; VIG-EX: vigorous intensity exercise group.

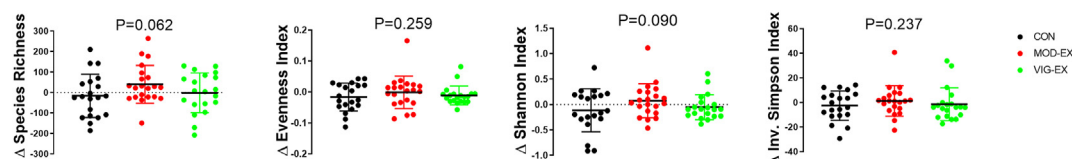


Fig. 3. Effect of a 24-week supervised concurrent exercise intervention on fecal microbiota diversity. Panel shows different indexes of fecal microbiota alpha diversity (species richness, evenness index, Shannon index and inverse Simpson index). Symbols * and + represent significant ($P < 0.05$) differences across groups after post-hoc Bonferroni corrections derived from one-way analyses of covariance adjusted for baseline values. Abbreviations: CON: control group; MOD-EX: moderate intensity exercise group; VIG-EX: vigorous intensity exercise group.

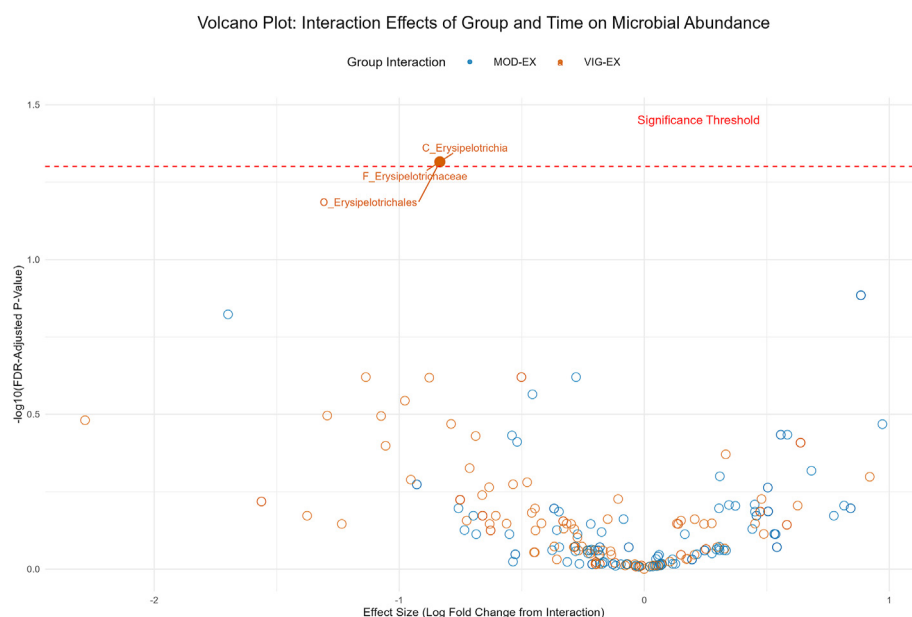


Fig. 4. Volcano plot showing the interaction effects of groups and time on microbial abundance. The x-axis represents the effect size ($\log_2$ fold change), and the y-axis indicates the $-\log_{10}$ of the FDR-adjusted p-value. The comparison is made with the reference group (CON) relative to the two intervention groups, MOD-EX and VIG-EX. Significant taxa (FDR-adjusted $P < 0.05$) are highlighted, with those enriched in the intervention groups appearing on the right and those enriched in the reference group on the left. This analysis highlights taxa that are differentially abundant as a function of both group and time interaction. Abbreviation: CON: control group; MOD-EX: moderate intensity exercise group; VIG-EX: vigorous intensity exercise group.

of uncovering taxa that might be associated with the observed metabolic and phenotypic changes after the intervention. In Fig. S2, illustrates the correlations between deltas in bacterial composition and deltas in body composition, metabolic, and dietary variables. Several strong and significant correlations ($R \geq 0.5$ or $R \leq -0.5$) were identified, suggesting that the exercise-induced changes may result from overall shifts in the microbial microenvironment rather than being solely driven by changes in a single bacterial genus.

4. Discussion

This study shows that a 24-week supervised concurrent exercise intervention, at moderate and vigorous intensities, does not modify fecal microbiota beta or alpha diversities in young adults. VIG-EX showed a decrease in the relative abundance of the *Erysipelotrichia* class, *Erysipelotrichales* order and *Erysipelotrichaceae* family, whereas the genus *Coprococcus* increased its relative abundance in MOD-EX. Additionally, the alterations in fecal microbiota composition were found to be independent of the changes in body composition induced by exercise.

Our results concur with previous research indicating that exercise does not influence beta and alpha diversities [29,51]. Specifically, an 8-week concurrence exercise program did not alter alpha diversity in sedentary adults aged 18–40 years [51]. Similarly, lean,

sedentary adults [28] and those with overweight/obesity [29] (20–45 and 32–40 years, respectively) showed no change in alpha diversity in response to similar exercise interventions. However, another study revealed that 24 weeks of aerobic exercise performed at vigorous intensity increases fecal alpha diversity by 5% in middle-aged adults with overweight/obesity [52]. Given that age and adiposity negatively correlate with alpha diversity [52], therefore, it is possible that long-term exercise only increases alpha diversity when initial levels are below normal [53]. This hypothesis is supported by our study's results, as all participants were young, and had normal fecal microbiota diversity indices fell before the study [54]. Further research is needed to elucidate the effective exercise dose, intensity, and frequency for enhancing fecal microbiota diversity in young adults.

In our study, the VIG-EX group showed a decrease in the relative abundance of the *Erysipelotrichaceae* family (*Bacillota* phylum) in fecal microbiota. This aligns with recent studies that reported a similar decrease in the relative abundance of the *Erysipelotrichaceae* family in the gut and a concurrent reduction in body weight in mice that voluntarily wheel running for wheels for 5–7 weeks [16,55]. We also found a decrease in the percentage of fat mass in the MOD-EX and VIG-EX groups after the intervention, but this reduction was not related to the decrease observed in the relative abundance of the *Erysipelotrichaceae* family (Fig. S2).

Bacteria belonging to the *Erysipelotrichaceae* family are implicated in the development of certain chronic diseases [56], and their abundance is positively associated with colorectal cancer [57] and obesity [58] in humans. High-fat or Western diets increase the relative abundance of the *Erysipelotrichaceae* in mice [59,60], while physical activity levels are negatively associated with the relative abundance of the *Erysipelotrichaceae* family in elderly men (≥ 65 years old) [61]. The specific role of these bacteria in host metabolism and the mechanisms through which exercise can modify their relative abundance in the gut warrant further investigation.

By analyzing the differential effects of exercise intensity on the fecal microbiota composition, we observed a distinct shift with an increase in the relative abundance of the *Coprococcus* genus in MOD-EX but not in VIG-EX. This finding is consistent with previous studies indicating that endurance training is associated with an enrichment of SCFA-producing bacteria, including *Coprococcus*, which has been associated with metabolic and inflammatory benefits [62,63]. Athletes generally have a different fecal microbiota composition than sedentary individuals, with higher levels of *Coprococcus* [21]. However, in our study, the increased fiber intake observed in MOD-EX may have contributed to the increase in *Coprococcus* genus, as fiber is an important substrate for SCFA-producing bacteria, reinforcing the interaction between diet and exercise in shaping the gut microbiota [64]. Previous studies have shown that higher fiber intake can increase butyrate production and support microbial diversity, particularly in response to exercise [19,65,66]. While these findings suggest a synergistic effect of exercise intensity and diet on microbiota composition, the mechanisms remain unclear.

Finally, the results of the exploratory analysis reflected in Fig. S2 suggest that microbial responses to exercise are likely the result of broader changes in the gut ecosystem rather than the isolated effect of a single bacterial genus. The fecal microbiota is highly dynamic, and exercise-induced changes in one bacterial group may influence the stability and function of others. For example, *Faecalibacterium* genus, a known butyrate producer with anti-inflammatory properties, showed a positive correlation with changes in fat mass in the MOD-EX group, suggesting that its abundance may be influenced by individual metabolic adaptations rather than exercise *per se* [67]. In contrast, the negative correlations observed for *Veillonellaceae* and *Verrucomicrobiaceae* genera with fat mass in the VIG-EX group are consistent with previous studies showing their enrichment in endurance athletes and their potential roles in metabolic efficiency and gut barrier function [68,69]. However, given the exploratory nature of these correlations and the limited significance of individual bacterial changes, further research is needed to determine whether these microbial changes contribute to or merely reflect exercise-induced metabolic adaptations.

4.1. Limitations and strengths

The results cannot be extrapolated to other populations since the study population only included young, sedentary adults. Furthermore, it is worth mentioning that fecal microbiota composition shows a high variability across individuals [70]. A limitation of the study is that stool sample collection was limited to a subset of participants, which could introduce selection bias if individuals more adherent to the intervention or with healthier lifestyles were overrepresented. Also, predicted pathways were inferred from the relative abundance of the microbial community identified by 16S rRNA sequencing, which has inherent limitations in resolving functional potential at high taxonomic resolution. In addition, direct metabolomic analysis of fecal samples was not performed, limiting our ability to confirm functional inferences derived from microbiota composition. Another limitation of this study is the absence of intermediate sampling points during the intervention

period, which restricts our ability to capture the dynamic trajectories of gut microbiota changes in response to exercise. On the other hand, our study has several strengths: (i) our participants are homogeneous in terms of age, health status, physical activity levels, and BMI; (ii) the study design is a randomized controlled trial, with a control group; (iii) we were able to investigate the dose–response effect of exercise on fecal microbiota diversity and composition; and (iv) we sequenced the fecal microbiota diversity and composition with *Illumina* platform and annotated with RDP.

5. Conclusions

A 24-week supervised concurrent exercise intervention performed at both moderate and vigorous intensity, slightly alters fecal microbiota composition without modifying fecal microbiota diversity in young adults. While the exercise interventions effectively reduced body fat, the observed changes in fecal microbiota composition were independent of the observed exercise-induced changes.

Author contributions

Borja Martínez-Tellez, Huiwen Xu, Lourdes Ortiz-Alvarez, Patrick C.N. Rensen and Jonatan R. Ruiz contributed to the study conception and design. Huiwen Xu, Lourdes Ortiz-Alvarez, Carmen Rodríguez-García, Milena Schöнке, Lucas Jurado-Fasoli, Francisco J Osuna-Prieto, Juan M.A. Alcantara, Francisco M. Acosta, Francisco J. Amaro-Gahete, Gert Folkerts, Ramiro Vilchez-Vargas, Alexander Link, Julio Plaza-Díaz, Angel Gil and Idoia Labayen performed material preparation and data collection and interpretation. Borja Martínez-Tellez, Huiwen Xu, Lourdes Ortiz-Alvarez, and Carmen Rodríguez-García performed the statistical analyses. All authors read, commented on, and approved the content of the manuscript. Borja Martínez-Tellez and Jonatan R. Ruiz had the primary responsibility for final content.

Data availability statement

Data are available from the corresponding author on reasonable request.

Ethics approval statement

Study protocol was approved by the Ethics Committee on Human Research of the University of Granada (nº.924) and Servicio Andaluz de Salud (Centro de Granada, CEI-Granada).

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

No generative AI or AI-assisted technologies were used in the creation of this manuscript. All writing, data analysis, and interpretation were conducted by the authors without the assistance of AI tools.

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Conflict of interest

The authors have no other conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.clnu.2025.04.008>.

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