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SHORT COMMUNICATION

Effects of 12 weeks of resistance training on rat gut microbiota composition

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ABSTRACT

In addition to its health benefits, exercise training has been noted as a modulator of the gut microbiota. However, the effects of resistance training (RT) on gut microbiota composition remain unknown. Wistar rats underwent 12 weeks of RT. Body mass, glucose tolerance, visceral body fat, triglyceride concentration and food consumption were evaluated. The gut microbiota was analyzed by 16S rRNA gene sequencing. Rats that underwent RT showed lower body mass (P=0.0005), lower fat content (P=0.02) and better glucose kinetics (P=0.047) when compared with the control. Improvements in the diversity and composition of the gut microbiota were identified in the RT group. The relative abundance of *Pseudomonas*, *Serratia and Comamonas* decreased significantly after 12 weeks of RT (P<0.001). These results suggest that RT has the potential to enhance the diversity of the gut microbiota and improve its biological functions.

KEY WORDS: Strength exercise, Intestinal microbiome, Metabolism, Animal model

INTRODUCTION

Exercise training is an essential component of a healthy lifestyle, and its beneficial effects have been well documented for years (Hawley et al., 2014). More recently, studies have demonstrated the role of exercise in modulating gut microbiota (Petriz et al., 2014; Barton et al., 2018). Evidence from animal models and humans supports the idea that exercise has a beneficial effect on gut microbiota structure and diversity (Carbajo-Pescador et al., 2019). Moreover, the gut microbiota produces/forms short-chain fatty acids (SCFAs) that act directly on the host's energy metabolism, mainly in skeletal muscle (Frampton et al., 2020).

Regarding exercise training, in animal models, the literature thus far has focused on aerobic exercise in a voluntary model (Matsumoto et al., 2008), forced on a treadmill (Petriz et al., 2014), or both (Allen et al., 2015). Thus, it is expected that exercise intensity and type may promote different physiological and gut microbial changes. In this sense, resistance training (RT) may exhibit a different stimulus for the gut microbiota and elicit differential effects as a result of adaptations to predominantly endurance or resistance stimuli (Coffey and Hawley, 2007; Petriz et al., 2017).

RT is beneficial for increasing muscle strength, functionality and muscle hypertrophy (Evans, 2019), preventing outcomes such as sarcopenia in elderly individuals (Aagaard et al., 2010; de Freitas et al., 2019). Although RT in animal models is well established (Santana et al., 2019; Padilha et al., 2019; de Sousa Neto et al., 2020), it has not yet been as deeply investigated as endurance training, especially with regard to changes in the gut microbiota.

Although several studies have reported a relationship between gut microbiota changes and muscle mass (Siddharth et al., 2017; Ticinesi et al., 2017; Ni Lochlainn et al., 2018), as well as an improvement in the expression of bacterial genes associated with catabolism in sarcopenic rats (Siddharth et al., 2017), no studies to date have provided knowledge about the gut microbiota in response to RT in rats. The current study aimed to investigate the effects of 12 weeks of RT on rat gut microbiota and metabolic parameters. We hypothesized that 12 weeks of RT would promote positive modulation in microbial diversity, improving metabolic function. Additionally, we expected that modulation would be different from that in models of endurance training, mainly concerning the relative abundance of taxa.

MATERIALS AND METHODS

Ethical approval

All procedures performed on animals were in accordance with international recommendations and ARRIVE (Animals in Research: Reporting *In Vivo* Experiments) guidelines (Percie du Sert et al., 2020). The animal research ethics committee approved the study (854/2017). Thus, the smallest, viable number of animals was used for the experiment. Animals were killed by intraperitoneal injection of ketamine/xylazine mix (Nogueira et al., 2020).

Animals and study design

Wistar male rats (*Rattus norvegicus*), 45 days old, 216.5±10.9 g (mean±s.d.), were allocated to collective cages (2–3 animals per cage) on a 12 h light–dark cycle within a thermo-controlled environment. Access to food and water was provided *ad libitum* (Reeves et al., 1993).

After an acclimation period of 2 weeks, the animals' gut microbiota was characterized (baseline). Afterward, the animals were randomized into two groups: sedentary control (control, n=5) and resistance training (RT, n=5). Body mass was recorded once per week, and 48 h after the last procedure at the end of the 12 week experiment. Analysis of the main outcomes was conducted between groups at the end of the experiment to avoid the 'time effect' that can modify animals' gut microbiota (Petriz et al., 2014).

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RT

The animals were adapted to the exercise equipment (vertical ladder) without load according to Almeida et al. (2020). The maximum carrying capacity (MCC) was ascertained before the training protocol. MCC consisted of progressive climbing, with 75% of the body weight of the animal. After each set, an additional 30 g was added to the charging apparatus until the animal was unable to complete the climbing movement (Nogueira et al., 2020). The RT protocol consisted of 12 weeks of training, 3 times a week, with at least four series with progressive loads of 50%, 75%, 90% and 100% of the MCC with 2 min of interval rest between each set. When the 100% load was reached, 30 g were added to the equipment until the rats stopped climbing the ladder. This protocol has been widely used in recent studies with RT in rodents (de Cássia Marqueti et al., 2017; Santana et al., 2019; Almeida et al., 2020; Nogueira et al., 2020).

Metabolic analysis

For glucose kinetics analysis, a glucose tolerance test (GTT) was performed 48 h after the end of the experimental period. After 6 h of fasting, a glucose solution was infused into the intraperitoneal region (50%, 2 g kg^{-1}), and blood levels were monitored at different times (15, 30, 60 and 120 min). Triglycerides were also analyzed at the same time points as fasting glucose. Analyses were carried out on tail blood samples using the Accutrend® Plus System (Roche). The visceral adipose tissue was determined by the sum of mesenteric, omental, perirenal and epididymal fat sites.

Stool sample collection

Three stool samples were collected from each animal before the experiment (baseline), and after 12 weeks of RT, totaling 45 samples. After each collection, samples were kept on ice and were subsequently frozen at −20°C until further analysis.

DNA extraction and 16S rRNA gene sequencing

About 0.22 g of each stool sample was used for DNA extraction using the QIAamp DNA Stool Mini Kit (Qiagen®) as per the manufacturer's instructions. The DNA extracted from each group (5 samples per group) was not pooled, and DNA concentration was determined by Qubit Fluorometric Quantification (Life Technologies®). The variable region V3–V4 of bacterial 16S rRNA genes was amplified. Primers used were 341F (5′-TCG-TCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGG-NGGCWGCAG-3′) and 805R (5′-GTCTCGTGGGCTCGGAGA-TGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′). A MiSeq benchtop sequencer performed amplicon sequencing with 2×250 base pair (bp) paired-end reads according to the standard protocol (16S Metagenomic Sequencing Library Preparation, Illumina).

Bioinformatics and statistical analysis

The sequences were processed using QIIME software package version 1.9.1 (Caporaso et al., 2010). Briefly, the sequences were filtered for quality control and grouped into operational taxonomic units (OTUs) using 97% similarity, using an open-reference OTU picking protocol to analyze α and β diversity. Thus, to avoid bias in subsequent analyses (α and β diversity), as a result of expected discrepancy values between regions of the sequence, it was necessary to normalize the samples, defining the number of minimal sequences for maximum utilization samples. Thus, the α and β diversity analysis was conducted with a rarified OTU table containing 40,000 sequence reads.

Bivariate relationships were measured with Pearson correlations and regression analysis. Samples were compared by ANOVA followed by Tukey and Bonferroni tests to compare means (taxonomic differences). For prediction, functional gene analysis samples were compared by Welch's *t*-test with Bonferroni correction using the software STAMP 2.0.0 (Parks and Beiko, 2010). Finally, functional genes were predicted based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways by PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved state) based on 16S rRNA sequencing data (Langille et al., 2013).

RESULTS AND DISCUSSION

At the end of experiment, the RT group had a lower body mass (Fig. 1A; $F_{4,32}$=6.765, P=0.0005). No differences were observed in food intake (Fig. 1B; $F_{1,16}$=2.208, P=0.156). Regarding glucose kinetics, two-way ANOVA did not reveal any interaction (groups×time) (Fig. 1C; $F_{4,16}$=1575, P=0.22). However, the RT group showed a lower area under curve (AUC) when compared with the control group (Fig. 1D, P=0.047), and a lower visceral adipose tissue content (Fig. 1E; P=0.019). No differences were observed in triglyceride concentration (Fig. 1F; P=0.38).

Sequencing resulted in 1,037,988 sequences, and after the quality filter steps, a total of 942,225 quality 16S rDNA sequences was obtained with an average length of 420 bp. A mean (±s.d.) of 62.815 ±5.57 reads were obtained per sample, which were assigned to 1302 OTUs. In the fecal samples, the reads corresponded to 25 phyla, 49 classes, 80 orders, 141 families, 294 genera and 121 species. At the phylum level, the microbial composition in all groups showed a prevalence of Firmicutes and Bacteroidetes, followed by Proteobacteria (Fig. 2A). At the genus level, a total of 294 genera were identified from all samples.

The OTU richness was estimated by Chao1 index, which is based on both the number and evenness of observed OTUs. The results showed a significant increase of Chao1 index (F=36.3, P<0.0001) after 12 weeks in the RT group (707.4±57.1) when compared with the control group (591.9±34.0), and with baseline samples (471.4 ±36.6). Collectively, most OTUs were shared among the three, with 19, 61 and 432 OTUs uniquely identified from the baseline, control and RT fecal samples, respectively (Fig. 2B).

To investigate the effect of RT on β diversity analysis, an unweighted UniFrac distance matrix was used. A principal components analysis (PCA) plot showing gut bacterial communities at the OTU level indicated a similar bacterial community structure between the baseline and control groups. However, the RT group was clustered separately, indicating that the intervention resulted in a different bacterial structure (Fig. 2C; analysis of similarities, P<0.05). These data indicate that RT did significantly alter the bacterial communities and affected the gut microbiota.

The difference in the changes in the relative abundance of taxa between the control and RT groups was evaluated by ANOVA, followed by the Tukey test (P<0.05) with Bonferroni correction for multiple comparisons (P<0.05). There were significant changes in the relative abundance of genera (Fig. 3A). The relative abundance of the *Pseudomonas*, *Serratia* and *Comamonas* genera significantly decreased in the RT versus the control group. In contrast, the relative abundance of *Coprococcus_1* was significantly increased in the RT group. Side-by-side comparison of the RT and control group (Fig. 3B) showed a significant decrease in KEGG annotated to lipid metabolism, metabolism of other amino acids, and cellular processes and signaling, and an increase in

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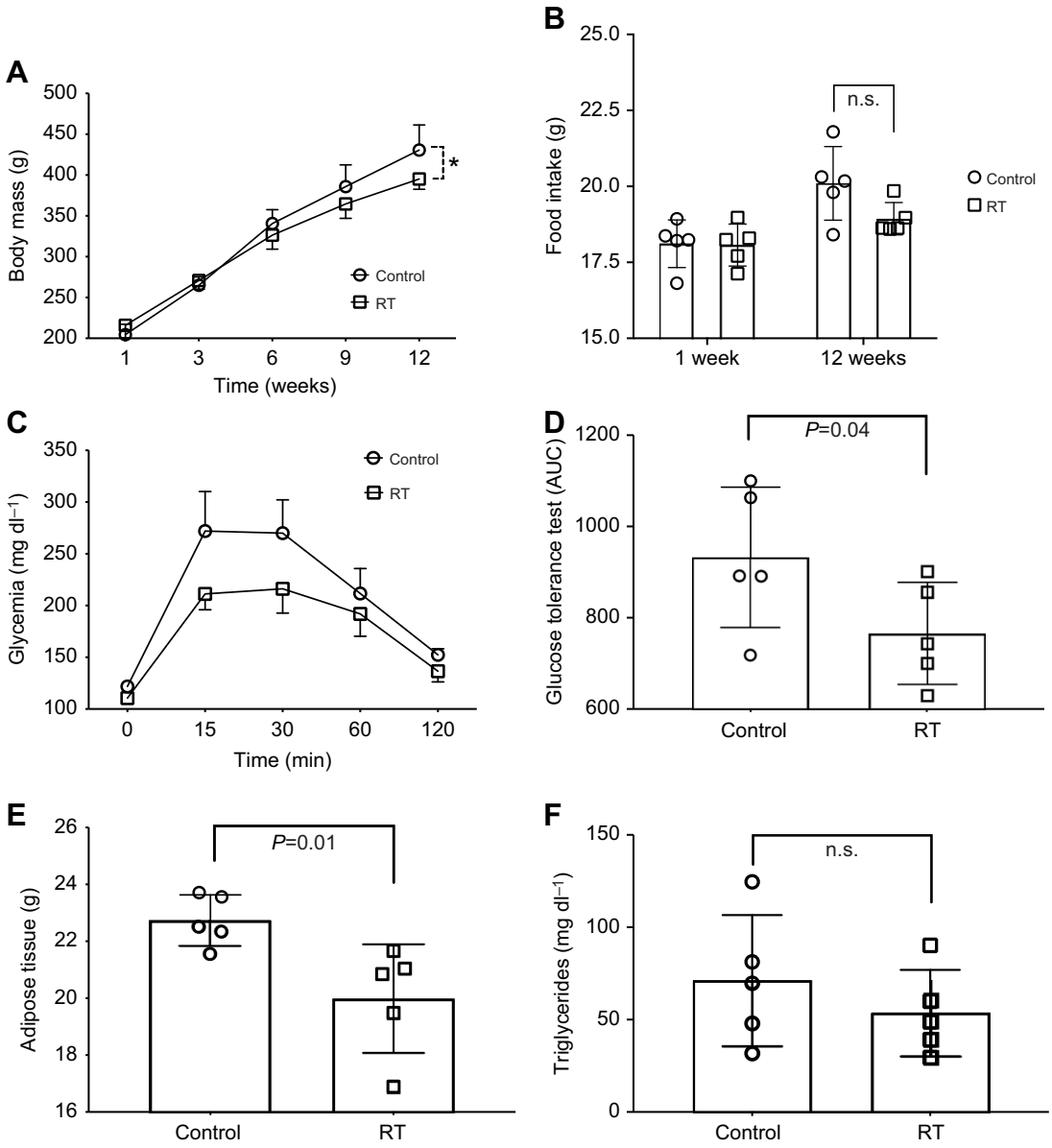

Fig. 1. Characteristics of the sedentary control and resistance training (RT) groups after 12 weeks. (A) Body mass kinetics. (B) Food intake. (C) Glucose kinetics. (D) Area under the curve (AUC) for the glucose tolerance test. (E) Visceral adipose tissue content. (F) Serum triglyceride concentration. *P<0.05 in relation to control group; n.s., not significant (P>0.05). Two-way ANOVA or t-test was used.

environmental adaptation after 12 h of RT (P<0.05). Similar to the increased gut microbial diversity observed following RT, microbiota-predicted functional pathways differed between groups.

To the best of our knowledge, this is the first study to examine the effects of RT and modulation of gut microbiota. Our findings indicate that RT reduced visceral fat and improved glucose metabolism, body mass control and muscle strength, and induced positive gut microbiota modulation in rats. Additionally, there was a pervasive effect of RT on gut microbiota community structure and an increase of bacterial alpha richness after 12 weeks. Increasing diversity has been related to health outcomes (Shanahan, 2010) that maintain crucial metabolic and structural functions. In contrast, low diversity is associated with pathological conditions such as obesity (Le Chatelier et al., 2013). Although the dietary pattern is notable, food intake changes can impact the modulation of the intestinal microbiota (Leeming et al., 2019). However, in the present study, there were no significant changes in food consumption between groups (P=0.156). Consequently, we conclude that RT regulated body mass gain and was a determinant in the remodeling of the gut microbiota of the animals investigated.

Regarding β diversity (PCA), the RT group demonstrated a different bacterial structure from the control condition (Fig. 2C), suggesting that RT affected the overall variation in gut bacteria. Thus, we investigated changes in the relative abundance of taxa between groups. Significant reductions occurred for *Pseudomonas*, *Serratia* and *Comamonas* following RT. A greater abundance of *Pseudomonas* is associated with inflammatory conditions and presents high pathogenicity (Shimizu et al., 2011). Increased levels of *Serratia* are also associated with aspects of immunomodulation, which are common in infections and hospitalized individuals (Sampson and Fisher, 1980). From this perspective, dysbiosis in fragile individuals is frequent, which is related to numerous factors such as physical inactivity (van Tongeren et al., 2005). In this sense,

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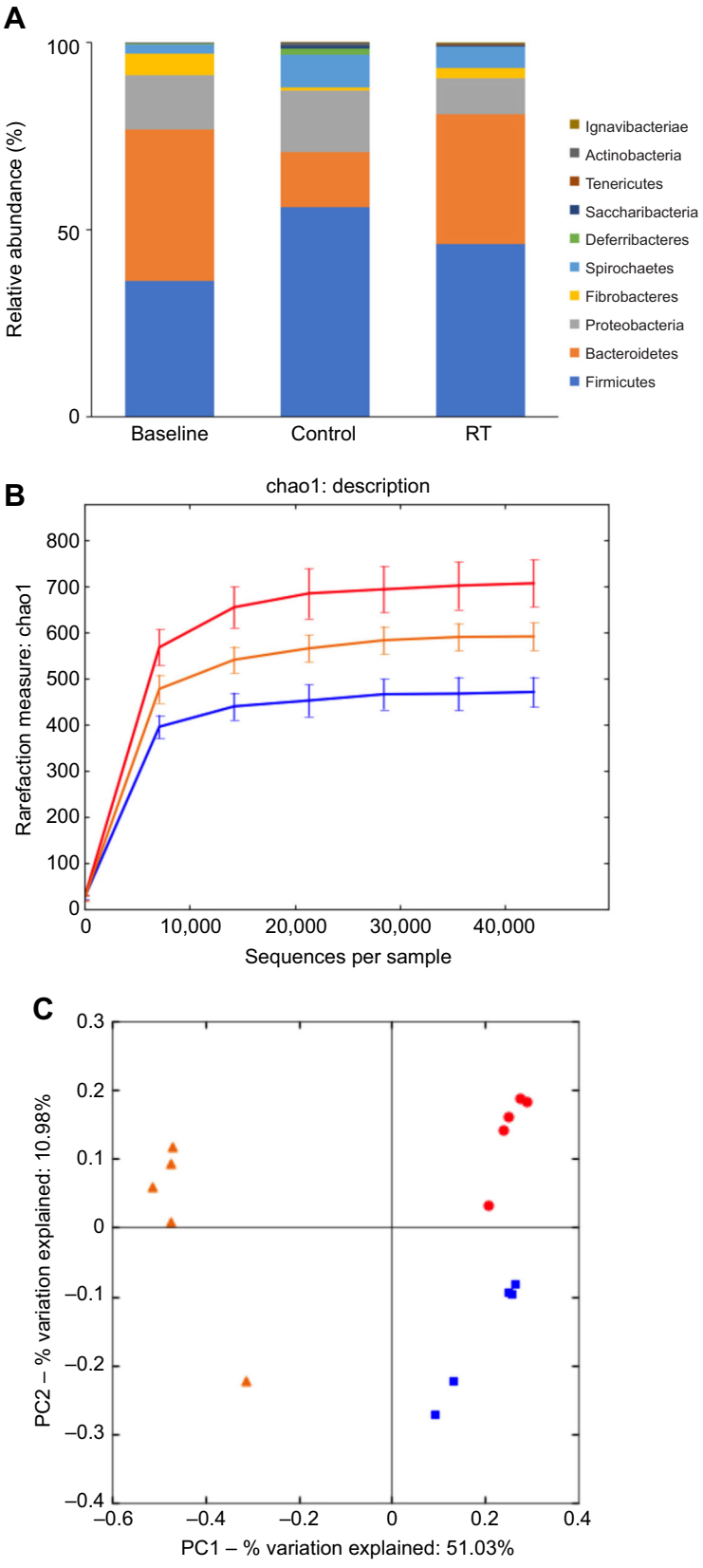

Fig. 2. Microbial composition of fecal samples from the experimental groups. (A) The relative abundance of the 10 most significant phylum frequencies from baseline, control and RT group fecal samples. (B) Rarefaction curves of the sequences of the 16S rDNA gene library. The data are expressed as means±s.d. Red line, RT group; orange line, control group; blue line, baseline. (C) Principal coordinates analysis (PCA) plot of the 16S rRNA gene amplicon sequenced samples. After 12 weeks, the RT group (orange) demonstrates substantial inter-individual variation from the control (blue) and baseline (red) groups.

exercise training is associated with eubiosis, showing that aerobic power levels are correlated with enhancing gut microbiota diversity, regardless of the type of diet (Estaki et al., 2016).

Recently, Renson et al. (2020) found that higher levels of *Pseudomonas* and *Serratia* are correlated with increases in levels of 'bad' lipids (e.g. LDL) and glucose. Additionally, *Serratia* was strongly associated with damage markers, an inflammatory process in aging. Accordingly, our results demonstrated that 12 weeks of RT were sufficient to decrease their relative abundance, indicating that this training modality can modulate the gut microbiota, particularly

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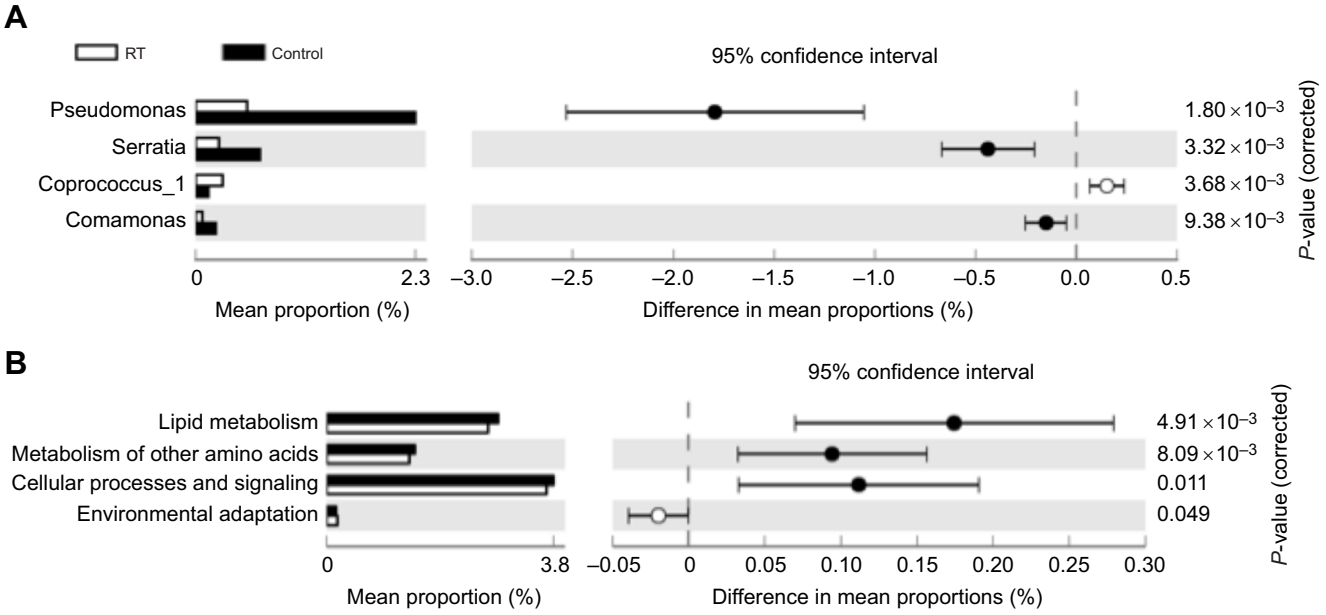

Fig. 3. Relative abundance of taxa and metabolic pathways of the experimental groups. (A) Effect of RT on bacterial relative abundance, obtained from statistical analysis of genus-level profiles. (B) Metagenome prediction of metabolic pathways from KEGG for the gut microbiota in the control and RT groups. These plots show the significant features with an α-value of <0.05. A two-sided *t*-test (equal variance) was carried out to test for taxonomic differences between control and RT samples using STAMP software version 2.0.0.

in rare *Proteobacteria* members and little studied in the context of the intestinal ecosystem.

Additionally, exercise training is effective in preventing and maintaining lean mass and helping to control glucose levels. The present study demonstrated an improvement in glucose control (Fig. 1C,D). In this sense, the findings of Liu et al. (2020) indicate the microbiota of mice that underwent exercise training showed better metabolic responses. Thus, a high abundance of *Coprococcus* was associated with a greater capacity for SCFA biosynthesis. Our results demonstrate an increase in *Coprococcus_1*, which may explain the improvement observed in glucose clearance. However, our findings have no cause–effect relationship. Nevertheless, exercise training is an essential tool in improving the concentration of GLUT4, which indicates an improvement in glucose metabolism (Cunha et al., 2015).

The effects of RT might be a vital driving force shaping microbial communities and resulting in functional differences. PICRUSt analysis has been widely employed for function predictions of gut microbiota (Denou et al., 2016; Taniguchi et al., 2018). However, at present, we still have limited knowledge of how exercise may induce changes in KEGG-assigned metabolic pathways. An alteration in lipid metabolism pathways was observed, evidenced by a reduction in visceral adipose tissue of the trained animals. However, the concentration of triglycerides was not reduced. This same behavior was observed by Stotzer et al. (2018), in which the RT protocol was not effective in causing differences in triglyceride levels, although they identified improvements in other parameters of lipid and glucose profiles. Regarding the signaling process and metabolism of other amino acids, a direct relationship between muscle hypertrophy and increases in strength levels has been shown (Nikooie et al., 2020).

Therefore, these changes in the predicted metabolic pathways should be interpreted with caution. However, considering RT as an essential tool in the maintenance of muscle mass, which promotes glycemic regulation and lipid reduction, it is suggested that the intestinal microbiota may have an influence on protein synthesis pathways as well as transcription factors, which are involved in the regulation of metabolism in different cellular processes. Although the present study has some limitations, such as not determining SCFAs, functional capacity (i.e. metagenomics) measures and longitudinal comparisons, our results suggest that RT enhances gut microbiota abundance and improves its biological functions. Although further study is needed to clarify the mechanism by which RT exerts an effect on the composition of the intestinal microbiota, our observations contribute to the initial basis for the investigation of these effects of RT on the gut microbiota.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: A.P.C., K.K.S., R.C.A., J.A.A.; Methodology: A.P.C., C.S.M., F.C.A., R.C.A., J.A.A.; Software: A.P.C., F.C.A., J.A.A.; Validation: A.P.C., F.C.A., J.A.A.; Formal analysis: A.P.C., K.K.S., F.C.A., R.C.A., J.A.A.; Investigation: A.P.C., K.K.S., C.S.M., J.A.A.; Resources: A.P.C., K.K.S., C.S.M., F.C.A., R.C.A., J.A.A.; Data curation: A.P.C., K.K.S., C.S.M., F.C.A., R.C.A., J.A.A.; Writing - original draft: A.P.C., K.K.S., C.S.M., F.C.A., R.C.A., J.A.A.; Writing - review & editing: A.P.C., K.K.S., C.S.M., J.A.A.; Visualization: A.P.C., K.K.S., C.S.M., F.C.A., R.C.A., J.A.A.; Supervision: A.P.C., K.K.S., R.C.A., J.A.A.; Project administration: A.P.C., F.C.A., R.C.A., J.A.A.; Funding acquisition: A.P.C., R.C.A., J.A.A.

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Data availability
The fastq files have been submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive database, as BioSample accessions: SAMN14730675, SAMN14730676, SAMN14730677.

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