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Probiotic Supplementation Induces a Partial Shift Toward an Active-Like Gut Microbiome in Physically Inactive Young Adults

Jiaru Yan^{1,2,3}, Feiyan Zhao^{1,2,3}, Zheng Sun⁴, Lai-Yu Kwok^{1,2,3}, Zhihong Sun^{1,2,3}, Wenyi Zhang^{1,2,3,*}, Meng Zhang^{1,2,3,*}

¹Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University, Hohhot 010018, China

²Key Laboratory of Dairy Products Processing, Ministry of Agriculture and Rural Affairs, Inner Mongolia Agricultural University, Hohhot 010018, China

³Inner Mongolia Key Laboratory of Dairy Biotechnology and Engineering, Inner Mongolia Agricultural University, Hohhot 010018, China

⁴Channing Division of Network Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115, USA

ABSTRACT: Sedentary behavior is associated with gut microbiota dysbiosis and increased metabolic risk, yet effective microbiome-modulating strategies for individuals who cannot engage in sufficient physical activity remain underexplored. Probiotics may modulate sedentary-associated microbial alterations. We conducted a cross-sectional multi-omics comparison of physically active (PA, $n = 65$) and physically inactive (PI, $n = 90$) young adults, followed by a 6-week randomized, double-blind, placebo-controlled trial in the PI cohort (probiotic: PIP, $n = 45$; placebo: PIC, $n = 45$). At baseline, PA individuals exhibited higher α -diversity and a more favorable microbial community structure than PI participants, enriched in *Bifidobacterium adolescentis*, *B. bifidum*, *Anaerostipes hadrus*, and associated metabolites. A random forest classifier distinguished PA from PI with high accuracy (96.1%; area under the curve, AUC = 0.993). Following intervention, PIP subjects showed a modest shift toward an active-like microbiome: root Jensen-Shannon similarity between PIP and PA (69.3%) was significantly higher than between PIC and PA (67.8%; $P < 0.001$), and levels of key taxa (*A. hadrus*, *B. bifidum*) and metabolites (vanillic acid, prolylglycine) moved closer to PA. Consistently, classifier performance decreased for PIP vs. PA (AUC 0.690; accuracy 68.2%) but remained higher for PIC vs. PA (AUC 0.843; accuracy 80.0%), indicating a probiotic-associated reduction in microbiome dissimilarity. These findings suggest that probiotic supplementation induces a partial, ecologically measurable shift toward an active-like gut microbiome configuration in inactive individuals, without replicating the full physiological benefits of regular physical activity, highlighting its potential as a scalable, adjunctive strategy for microbiome health in sedentary populations.

Keywords: Physical activity; Sedentary behavior; Gut microbiome; Gut metabolite; Probiotic intervention

1.1 Background

Sedentary behavior is increasingly prevalent and is consistently linked to gut microbiota dysbiosis and elevated metabolic risk, highlighting the need for accessible strategies to support gut ecosystem health in individuals unable to increase physical activity^[1]. The gastrointestinal microbiome is a complex and

*Corresponding author
zhangmeng1613@163.com; zhangwenyizi@163.com

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dynamic ecosystem within the human digestive tract comprising trillions of microorganisms, including bacteria, archaea, viruses, and fungi^[2-4]. It shows substantial variation among individuals and populations, influencing the immune system to different degrees in states of equilibrium^[2, 5, 6]. Physical activity is an effective lifestyle intervention that has a profound effect on gut health^[7, 8], and it can regulate the gut microbial community through diverse mechanisms like altering gut permeability, modulating immune responses, and transforming the composition and functionality of the gut microbiota^[8, 9]. The effect of exercise on gut health and immune function has been metaphorically described as a “J-curve” effect, implicating that moderate exercise can help optimize the balance of the gut microbiome, while excessive exercise may have adverse consequences^[7, 9].

Individuals who engage in regular, intensive physical activity differ markedly from those with chronically sedentary lifestyles in athletic performance, recovery capacity, and overall health status. These differences arise not only from training load and genetic background but also from the structure and function of the gut microbial ecosystem^[7]. Specifically, regularly active individuals tend to exhibit greater microbial α -diversity and enrichment of health-promoting taxa^[10]. However, many people are unable to substantially increase activity in the short term due to academic or occupational constraints. This reality underscores an urgent need for pragmatic adjunctive interventions to ameliorate sedentary-associated dysbiosis.

Probiotics are defined as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host”^[11]. They have been shown to remodel gut microbial composition, strengthen intestinal barrier function, and promote the growth of beneficial commensals^[12-14]. Among candidate strains, *Lactocaseibacillus rhamnosus* Probio-M9 (Probio-M9), originally isolated from human colostrum, has shown promise in modulating gut microbiome and metabolome profiles in diverse contexts^[4, 15, 16]. In animal models, Probio-M9 attenuates exercise-induced muscle inflammation and oxidative stress and enhances stress resilience^[17, 18]. Human studies report that it helps maintain microbiome stability during environmental stressors (e.g., prolonged sea voyages), improves quality of life in stressed adults, and favorably influences microbiome-immune-metabolic interactions in individuals with metabolic conditions^[4, 19, 20]. These attributes support its evaluation as a targeted intervention for sedentary-associated dysbiosis. However, evidence remains limited on whether probiotic supplementation can shift the gut microbiome of insufficiently active individuals toward features observed in regularly active counterparts, without implying equivalence to the full physiological effects of exercise.

To address this, we enrolled physically active (PA) and physically inactive (PI) young adults from a university-based cohort. We first conducted a cross-sectional, multi-omics comparison of taxonomic, functional, and metabolite profiles between PA and PI groups. Within the PI cohort, we then implemented a 6-week randomized, double-blind, placebo-controlled trial to test whether Probio-M9 supplementation induces a partial shift toward an active-like gut microbiome configuration. Our primary objectives were: (1) to define a multi-omics atlas of activity-associated differences in the gut ecosystem, and (2) to assess whether probiotic intervention promotes measurable, albeit partial, ecological alignment with the PA

microbiome. This work provides empirical insight into a scalable, non-exercise-based strategy to support gut microbiome health in sedentary populations, while clarifying the boundaries of what probiotics can, and cannot, achieve relative to physical activity.

1.2 Methods

1.2.1 Study design and participants

This study was conducted in a university-based adult cohort of young adults in Inner Mongolia, China. Participants were recruited through on-campus enrollment and online outreach and included predominantly undergraduate and graduate students, along with a small number of university staff. All participants provided written informed consent, and the study was approved by the institutional ethics committee.

The study comprised two integrated components: (1) A cross-sectional multi-omics comparison between PA and PI groups to characterize differences in gut microbial taxonomy, functional potential, and metabolite profiles; (2) A 6-week randomized, double-blind, placebo-controlled trial within the PI cohort^[4], re-evaluated here to test whether probiotic supplementation shifts the gut microbiome toward an active-like configuration relative to the PA reference.

1.2.2 Cross-sectional multi-omics comparison between PA and PI groups

At enrollment, all participants completed a standardized, structured self-report assessing demographic characteristics and habitual physical activity and sedentary behavior over the past 7 days. Physical activity status was classified using prespecified criteria: PA group ($n = 65$) comprised newly recruited individuals reporting ≥ 2 h/day on ≥ 6 days/week; PI group ($n = 90$) included individuals reporting > 8 h/day of sedentary time on ≥ 6 days/week and ≤ 2 structured exercise sessions per week, each lasting < 30 min from a previous study^[4]. We acknowledge that activity classification relied on self-report, which may be subject to misclassification; objective measures (e.g., accelerometry) were not collected in this phase.

For the 65 PA participants newly recruited for this study, stool was self-collected using the Longseegen stool storage kit (Guangdong Longsee Biomedical Co., Ltd., Guangzhou, China), stored at -80°C , and subjected to shotgun metagenomic sequencing and untargeted metabolomics. For the 90 PI participants, fecal metagenomic and metabolomic datasets generated in prior study were used with consistent laboratory protocols as reported therein^[4]; all samples in the current work were processed through a unified bioinformatics workflow to ensure comparability across datasets.

1.2.3 Probiotic intervention within the PI cohort

A 6-week randomized, double-blind, placebo-controlled trial was conducted within the PI cohort^[4]. Participants were randomly assigned to receive either probiotic supplementation (PIP, $n = 45$) or placebo (PIC, $n = 45$). Randomization was performed using a computer-generated sequence, and allocation concealment was maintained via coded, pre-packaged sachets prepared by an independent third party.

Both probiotic and placebo sachets were identical in appearance, taste, color, and weight to ensure blinding of participants, study personnel, and outcome assessors throughout the trial. Participants consumed

one sachet daily after breakfast for 6 weeks. The probiotic formulation delivered 5.0×10^{10} CFU/day of *Lactaseibacillus rhamnosus* Probio-M9. The placebo consisted of maltodextrin and strawberry powder, with no live microorganisms. Lifestyle data, together with stool samples, were collected at pre-intervention (week 0), and week 6. At each sampling time point, participants self-collected stool using standardized fecal collection tubes pre-loaded with a fecal microbiota DNA preservative. After defecation, the stool was transferred into the tube, thoroughly mixed with the preservative, and the samples were stored at -80°C in a medical cryogenic freezer until subsequent analyses.

To minimize dietary confounding, all participants received standardized dietary guidance encouraging consistent intake patterns during the intervention. However, detailed dietary intake was not quantitatively assessed, representing a limitation. Adherence was monitored through scheduled follow-ups and completion records. Adverse events, particularly gastrointestinal symptoms and general discomfort, were recorded at each follow-up. All analyses were conducted on a per-protocol basis, including only participants who completed the 6-week intervention and provided post-intervention stool and metadata.

1.2.4 DNA extraction, shotgun metagenomic sequencing, and data processing

The fecal metagenomic DNA was extracted using the QIAGEN DNA Stool Mini-Kit (Qiagen, Hilden, Germany) from 4.5 ml of stool suspensions, strictly following the manufacturer's guidelines. The quality and concentration of the extracted genomic DNA were carefully evaluated with gel electrophoresis and a Nanodrop 8000 (Thermo Scientific, Waltham, MA, USA). Two criteria were used to determine the quality of the metagenomic DNA: a concentration of at least 100 ng/mL and an absorbance ratio of 260 nm to 280 nm between 1.8 and 2.0. Qualified DNA were subjected to polymerase chain reaction. The PCR products of 0.2 μg DNA per sample were purified using the AMPure XP system (Beckman Coulter, Inc., Brea, CA, USA).

DNA libraries were prepared with an approximate fragment length of 350 bp using the NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA, Catalog #: E7370L), adhering strictly to the manufacturer's instructions. Index codes were added for sample identification. The DNA fragments underwent end polishing, A-tailing, and ligation with full-length adapters for Illumina sequencing, followed by additional PCR amplification. The final PCR products were again purified using the AMPure XP system. Subsequently, the quality of DNA libraries was rigorously evaluated on the Agilent 5400 Fragment Analyzer system (Agilent Technologies Inc., Santa Clara, CA, USA) and quantified by QPCR to target a concentration of 1.5 nM. For sequencing, 150 bp paired-end reads were generated in both the forward and reverse directions. The metagenomic sequencing was performed on an Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA), as described in previous studies^[21].

Raw sequencing data were subjected to quality control (QC) using fastp (v0.23.1)^[22] to eliminate low-quality reads and adapter sequences. To filter out human DNA, we used bowtie2^[23] (v2.4.4) to align the reads to the human GRCh38 genome. This rigorous process yielded an average of 22,089,890 high-quality, clean reads per sample, providing a robust dataset for further analysis.

For comprehensive taxonomical and functional profiling, the high-quality clean reads were analyzed using MetaPhlAn4^[24] (software version 4.0.6, database version vOct22_CHOCOPhlAnSGB_202212) and HUMAnN3^[25] (software version 3.6, database version v201901, available at <https://github.com/biobakery/humann>). MetaPhlAn4 was employed for generating a detailed taxonomic profile of bacteria, fungi, and archaea^[26]. Meanwhile, HUMAnN3 was employed for functional profiling of the microbial community. Both tools were run with their default parameters to ensure consistency and reliability of the analysis.

To profile the viral composition within our samples, we employed a de novo assembly approach according to the protocol outlined by Shang et al., using PhaBox^[27] (<https://github.com/KennthShang/PhaBOX>) on Linux systems. The process involved several key steps^[28]: (1) Assembly of metagenomic reads: MEGAHIT^[29] (v1.2.9) was used to assemble the metagenomic short clean reads from each individual into contigs. (2) Contig circularity assessment: The circularity of assembled contigs was assessed, with a focus on those larger than 3 kb in size^[27]. (3) Phage contig identification and annotation: From the assembled contigs, phage contigs were identified and subsequently annotated to the family level^[27]. (4) Phage contig construction: Phage contigs were constructed by clustering phage genomes with a > 95% identity^[30] threshold using MMseqs2^[31] (Release 13-45111). (5) Quantification of viral abundances: To quantify viral abundances, an index based on the clustered phage genomes was built using bwa-mem2^[32] (v2.2.1). The reads per kilobase per million (RPKM) for each sample were calculated using CoverM (v0.6.1, available at <https://github.com/wwood/CoverM>).

1.2.5 Metabolomic profiling and data processing

A standardized methodology was used to extract of metabolites from collected fecal samples^[33–35]. First, 25 mg of fecal sample was accurately weighed and subjected to solvent extraction with a mixture of methanol, acetonitrile, and water (in a 2:2:1 V/V ratio) with isotopically labeled internal standards^[35]. To ensure thorough extraction, the sample was mechanically disrupted at 35 Hz for 4 minutes, followed by ultrasonication in an ice water bath for 5 minutes. This process was repeated three times. Subsequently, the sample was allowed to stand at -40 °C for 1 hour to facilitate the precipitation of metabolites. The supernatant was collected after centrifugation at $13,800 \times g$ for 15 minutes at 4°C and used for further analysis. To ensure the instrumental stability of the analytical process, equal volumes of supernatant from all samples were pooled to create a QC sample.

Chromatographic separation of target compounds was achieved using a Vanquish ultra high-performance liquid chromatography system (Thermo Fisher Scientific, Inc., Waltham, MA, USA) coupled to a Waters ACQUITY UPLC BEH Amide column (2.1 mm $\times$ 100 mm, 1.7 μ m; Waters Corporation, Milford, MA, USA)^[36]. The mobile phase consisted of two components: phase A, an aqueous phase with 25 mmol/L ammonium acetate and 25 mmol/L ammonia solution, and phase B, acetonitrile. During the analysis, the sample tray temperature was maintained at 4 °C, and the injection volume was set at 2 μ L. Mass spectrometric analysis was conducted using a Thermo Scientific Orbitrap Exploris 120 mass

spectrometer controlled by Xcalibur software (v4.4). This setup facilitated both primary and secondary mass spectral data acquisition^[36].

Raw files were converted to mzXML format using ProteoWizard^[37] and processed in MS-DIAL (v4.9.221218) for peak detection, deconvolution, retention-time alignment, and gap filling. Features with intensity < 1,000 or detected in fewer than 50% of samples within at least one sample group were excluded. To ensure analytical reproducibility, features with > 30% relative standard deviation in QC samples were removed^[38]. The resulting dataset was normalized and transformed in MetaboAnalyst 5.0^[39] using median normalization, log₁₀-transformation, and auto-scaling.

Metabolite annotation was conducted in accordance to the Metabolomics Standards Initiative (MSI) framework. Putative identifications were based on accurate mass and MS/MS spectral matching against the Human Metabolome Database (HMDB); retention time was used when available. All reported metabolites are classified as MSI Level 2 (putatively annotated compounds). No authentic chemical standards were used, so Level 1 (confirmed structure) identifications were not achieved in this untargeted discovery workflow. Annotated features were mapped to Kyoto Encyclopedia of Genes and Genomes (KEGG) compound database for pathway-level interpretation.

1.2.6 Statistical analysis

Statistical analyses were performed using R (v4.0.5) and GraphPad Prism (GraphPad Software, San Diego, CA; <https://www.graphpad.com>). Alpha diversity (Simpson's index for diversity; Chao1 for richness) was computed for bacterial, viral, and functional profiles using Parallel-Meta Suite v3.5^[40]. Group comparisons (PA vs. PI; PIP vs. PIC) are reported as median values with interquartile ranges, and significance was assessed using the Wilcoxon rank-sum test (two-sided, $P < 0.05$). Beta diversity was quantified using root Jensen-Shannon distance (rJSD), calculated via the *vegdist* function in the *vegan* R package. Principal coordinate analysis (PCoA) was applied to the rJSD matrices for visualization. To assess group-level separation, rJSD similarity (defined as $1 - \text{rJSD}$) between all individuals across groups (e.g., PIP-PA vs. PIC-PA) was calculated, with distances between group centroids in PCoA space as a secondary summary measure. Additionally, permutational multivariate analysis of variance analysis (PERMANOVA; 999 random permutations) was used to test for significant clustering by group. Differential abundance of microbial taxa, functions, and metabolites was identified using a dual-filtering strategy: Wilcoxon rank-sum test (univariate, non-parametric) and multivariable linear modeling, adjusting for potential confounders (MaAsLin2)^[41]. In both methods, P -values were adjusted for multiple testing using the Benjamini-Hochberg procedure. Following MaAsLin2 conventions, features with FDR-adjusted $Q < 0.25$ and raw $P < 0.05$ ^[41] were considered exploratory signals. Features detected by both methods were prioritized to reduce false positives. All such findings are framed as hypothesis-generating, not definitive biomarkers. Co-occurrence networks were constructed using iNAP^[42], based on Spearman correlations between bacterial taxa, phages, metagenomic functions, and metabolites. Networks were filtered to include only features present in $\geq 50\%$ of samples within a group, with $|r| \geq 0.4$ and $P < 0.05$ ^[28, 42]. A random forest (RF) classifier (*randomForest*

R package) was trained to distinguish PA from PI (baseline) and post-intervention groups (PIP vs. PIC vs. PA), using taxonomic and functional features as input^[21, 43].

Data visualization was achieved using R packages, including ggplot2, ggpubr, and pheatmap for boxplots, scatter plots, and line charts. Categorical variables were compared using chi-squared tests in Prism.

1.3 Results

1.3.1 Regular physical activity influenced the host gut microbiome and metabolome structures

Regular physical activity has profound impact on the function and physiology of the host gut microbiota^[8, 44, 45]. Individuals who engage in regular physical activity exhibit higher levels of α -diversity within their gut microbiota, indicating a healthier and more diverse microbial composition. Conversely, changes in the β -diversity of the gut microbiota have been linked to the progression of various diseases^[2, 46, 47]. This correlation has generated interest in investigating how routine physical activity affects the composition, function, and metabolites of the gut microbiota, unveiling underlying mechanisms that regulate gut health and overall well-being.

To enhance the reliability of our research outcomes and minimize the potential impact of unknown confounding factors, we confined further gut microbiome and metabolome analyses to 155 young adults from Inner Mongolia Agricultural University. This narrowed down the participant pool and allowed us to control variables such as geographic location, occupation, age, diet, and lifestyle habits. The PA group included 65 newly recruited individuals who reported engaging in moderate-to-vigorous physical activity for at least two hours per day on at least six days per week. In contrast, the PI group consisted of 90 young adults who typically spent more than eight hours sitting on at least six days a week and engaged in exercise no more than twice a week, with each session lasting less than thirty minutes.

Our study conducted a comprehensive comparison between the PA and PI groups to explore the diversity and richness of their gut microbiome structure on the phylum, species, phage, and KEGG pathway levels based on the Simpson's diversity and Chao1 indices (Fig. 1a). At the phylum level, the PA group exhibited significantly higher Simpson diversity (median = 0.522) than the PI group (median = 0.497; $P < 0.001$, Wilcoxon rank-sum test; Fig. 1a). However, no significant differences were observed between the two groups at finer taxonomic levels, including class, order, family, genus, species, and strain levels ($P > 0.05$, Wilcoxon rank-sum test; Fig. 1a, Fig. S1a).

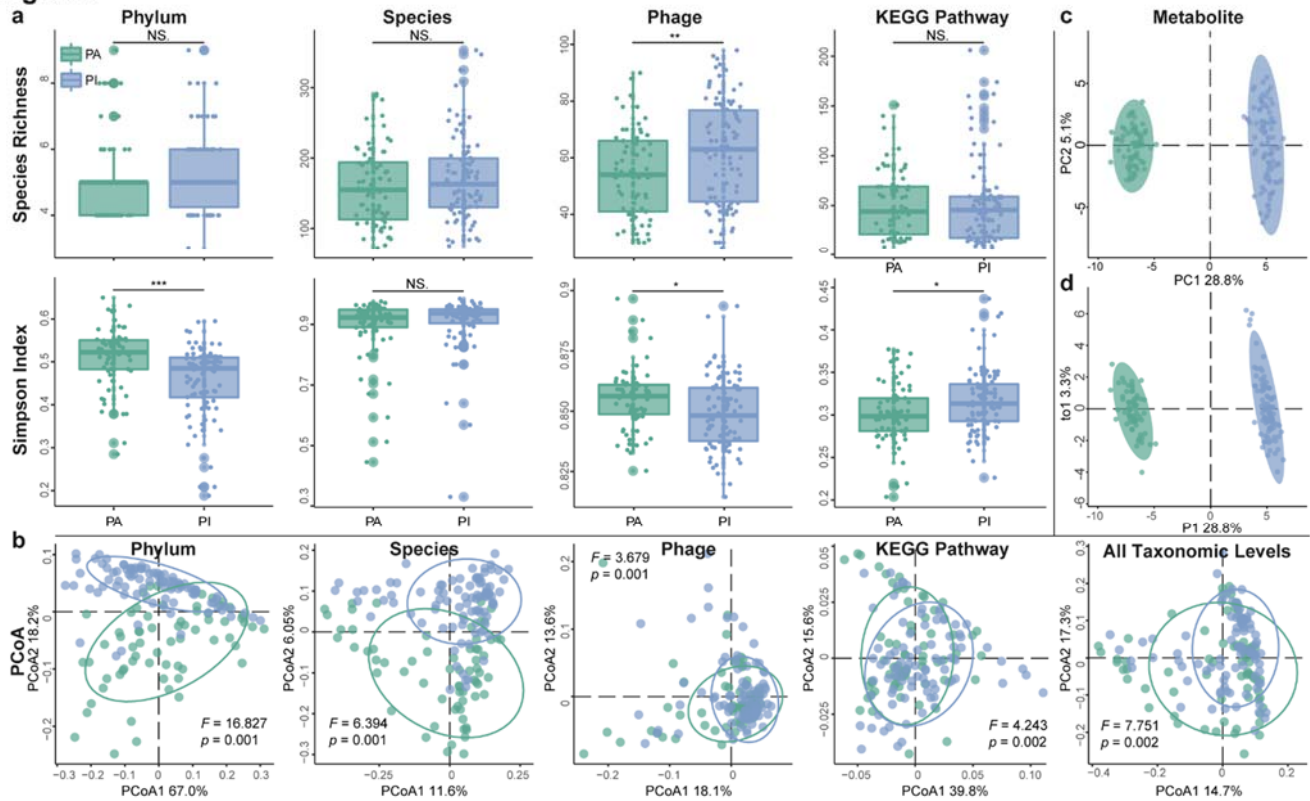
Figure1

Figure 1. Gut microbiome and metabolome differ between physically active (PA) and physically inactive (PI) young adults. (a) Alpha diversity of fecal microbial communities at the bacterial phylum, species, phage, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway levels. Top row: species richness (Chao1 index); bottom row: diversity (Simpson index). PA = green; PI = blue. P -values (Wilcoxon rank-sum test) compare PA vs. PI at each level. (b) Principal coordinate analysis (PCoA) based on root Jensen-Shannon distance (rJSD) at phylum, species, phage, KEGG pathway, and aggregated taxonomic levels. Ellipses indicate 95% confidence regions around group centroids. Permutational multivariate analysis of variance (PERMANOVA) F and P -values indicate significance of between-group separation. (c, d) Principal component analysis and orthogonal partial least-squares discriminant analysis of untargeted LC-MS/MS fecal metabolite profiles, intergroup separation. Statistical significance (Wilcoxon rank-sum tests): * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, $P > 0.05$.

Phages are viruses that infect bacteria, coexisting and co-evolving with their bacterial hosts, influencing the functionality of the entire gut environment^[48, 49]. Phages are involved in shaping the gut bacterial communities, affecting the host's immune system and metabolism, potentially contributing to disease progression^[49, 50]. Hence, we also analyzed the differences in the phage diversity between the PA and PI groups (Fig. 1a). Our results showed that the PA group had significantly higher phage diversity (Simpson index; median = 0.856 vs 0.848, $P = 0.013$) but lower phage richness (Chao1 index; median = 54 vs 63, $P = 0.0067$) compared to the PI group. In contrast, the PA group showed significantly lower functional diversity based on KEGG pathway analysis (Simpson index; median 0.298 vs 0.322, $P = 0.014$).

To assess the differences in β -diversity of the fecal microbiome between the PA and PI groups (Fig. 1b), PCoA was employed to visualize the structural differences in various levels of fecal microbiome features. The analysis revealed significant differences in fecal bacterial phylum, species, phage, KEGG pathway, and all bacterial taxa profiles (confirmed by the PERMANOVA test, with F values of 16.827, 6.394, 3.679, 4.243, and, 7.751; $P < 0.05$ in all cases; Fig. 1b). Furthermore, both principal component analysis (Fig. 1c) and

orthogonal partial least squares-discriminant analysis revealed a substantial difference in the fecal metabolomic structure between the PA and PI groups (Fig. 1d).

Collectively, these findings suggest that regular physical activity can influence the structure and function of the gut microbiome and metabolome, thereby modulating the overall health of the host. These observations highlight the relationship between lifestyle choices and the dynamic nature of the gut microbiome.

1.3.2 Microbiome and metabolome features associated with physical activity

1.3.2.1 Significant differential microbiome and metabolome signatures between PA and PI individuals

To accurately identify microbial markers associated with regular physical activity, we conducted differential abundance analysis on the fecal microbiome and metabolome at various levels between the PA and PI groups. The fecal microbiome analysis was achieved with a dual-method approach through integrating the Wilcoxon rank-sum test and MaAsLin2^[51]. This approach could enhance the precision and reliability of the findings, effectively pinpointing the microbiome markers that correlate with regular physical activity.

Our analysis revealed significant differences in the gut microbiome composition associated with regular physical activity. We identified two significantly differential phyla, 27 species, three phages, and five key KEGG metabolic pathways (Fig. 2a, Table S1) along with 80 distinct metabolites (Fig. 2b, Table S2). At the phylum level, the fecal microbiome of the PA group had significantly more Actinobacteria ($P < 0.05$). Additionally, the most dominant gut phylum in the PA group, *Firmicutes*, was less abundant compared to the PI group (PA group: $49.13\% \pm 16.06\%$; PI group: $59.17\% \pm 16.41\%$; $P < 0.05$; Fig. 2c). At the species level, 20 fecal bacterial species were significantly enriched in the fecal microbiota of the PA group, such as *Faecalibacterium prausnitzii*, *Blautia* (Bl.) *wexlerae*, *Bl. obeum*, *Bl. faecis*, *Anaerostipes hadrus*, *Bifidobacterium* (B.) *adolescentis*, *B. longum*, *B. pseudocatenulatum*, *B. bifidum*, and *Streptococcus salivarius*, compared to that of the PI group. Conversely, seven species, such as *Bacteroides fragilis*, and *Eubacterium ventriosum*, showed an opposite trend of change ($P < 0.05$; Fig. 2c).

Analysis of subjects' fecal phageome revealed that five different KEGG metabolic pathways, including K02874 and K02948, were significantly enriched in the PA group compared to the PI group ($P < 0.05$; Fig. 2a, Table S1). Fecal metabolites are direct mediators of the gut microbial activity that interact with the host. Comparative analysis of the fecal metabolome revealed 42 significantly enriched and 38 significantly depleted metabolites in the PA group compared with the PI group ($P < 0.05$; Fig. 2b, Table S2).

These differentially abundant gut microbiome and metabolome biomarkers may be associated with regular physical activity, playing a role in modulating the gut microbiome and host health status.

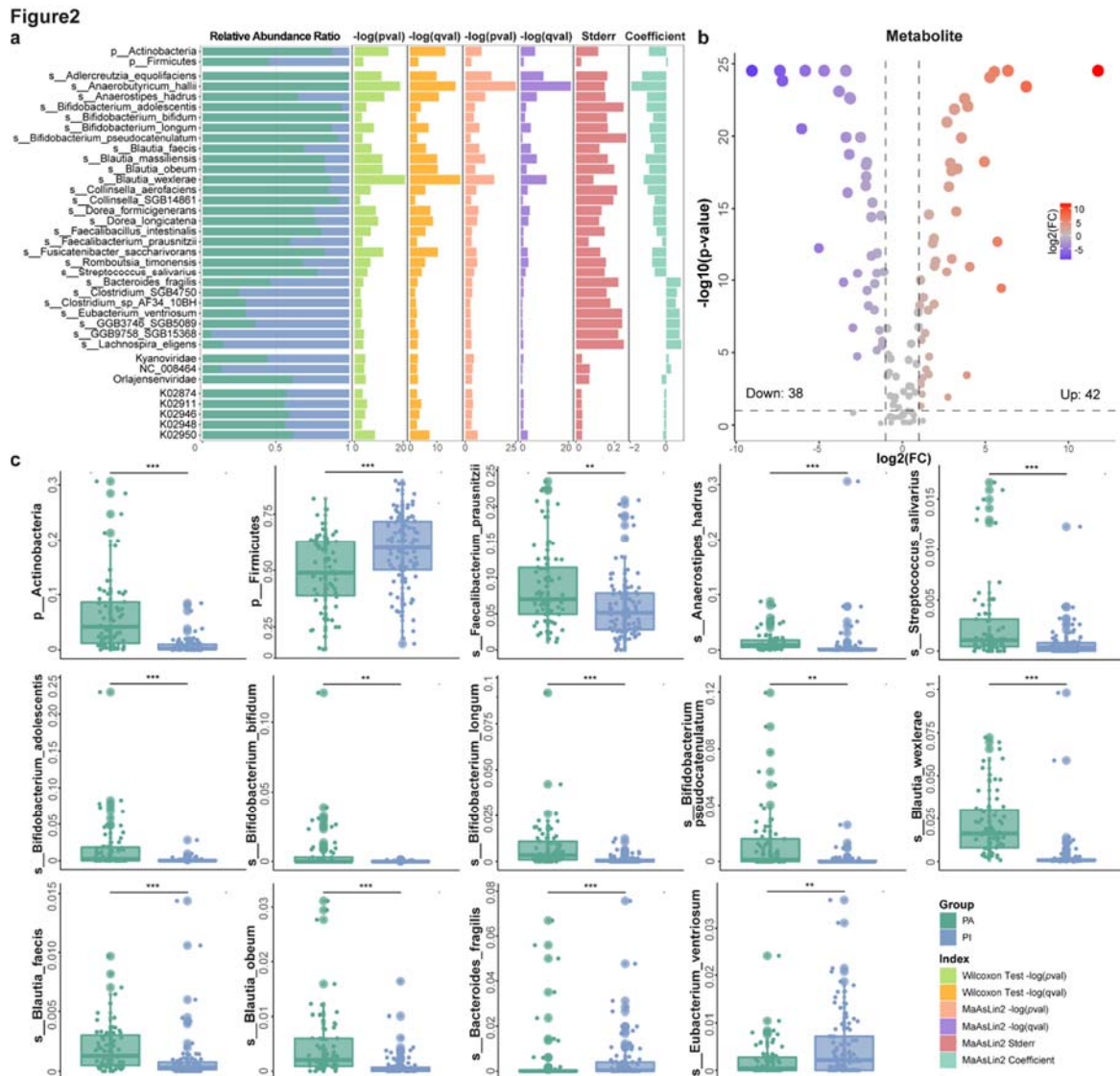


Figure 2. Differential microbial and metabolite features associated with physical activity status. (a) Relative abundance ratio (PA/PI) of bacterial taxa, phages, and KEGG pathways identified as exploratory differential features by Wilcoxon rank-sum test and Microbiome Multivariable Association with Linear Models (MaAsLin2; Benjamini-Hochberg FDR, $q < 0.25$ and raw $P < 0.05$). (b) Volcano plot of fecal metabolites (PA vs. PI). Each dot represents one metabolite; significantly differential metabolites meet $|\log_2(\text{fold change})| > 1$, variable importance in projection (VIP) scores > 1 , and FDR-adjusted $q < 0.05$. Color scale indicates $\log_2(\text{fold change})$; numbers denote counts of increased (up) and decreased (Down) metabolites. (c) Boxplots of representative PA- or PI-associated species selected from panel (a) and prior literature. Boxes show medians and interquartile ranges, with individual samples overlaid as dots. Significance level: $**P < 0.01$; $***P < 0.001$ (Wilcoxon rank-sum test).

1.3.2.2 Fecal microbiome and metabolome co-occurrence networks of PA and PI groups

To visualize the interconnectedness of the fecal microbiome and metabolome of the PA and PI groups, co-occurrence networks were constructed at the fecal bacterial species, phage, KEGG pathway, and metabolite levels (Fig. 3a, Fig. S2). A threshold of feature prevalence $> 50\%$, $P < 0.05$, and $|r| \geq 0.4$ was implemented in the network construction. The node count, edge count, average degree of connectivity, and network density were calculated and presented. Differences in these parameters reflect potential associations or interactions between specific fecal components that are influenced by physical activity levels. Notably, at the species level, the co-occurrence network of the PA group exhibited a higher average connectivity and network density compared to the PI group, indicating a more connected and dense microbial community in

the gut of individuals who engage in regular physical activity. However, at the levels of phage, KEGG metabolic pathway, and metabolite, the co-occurrence networks of the PI group showed a higher average connectivity and density.

Figure 3

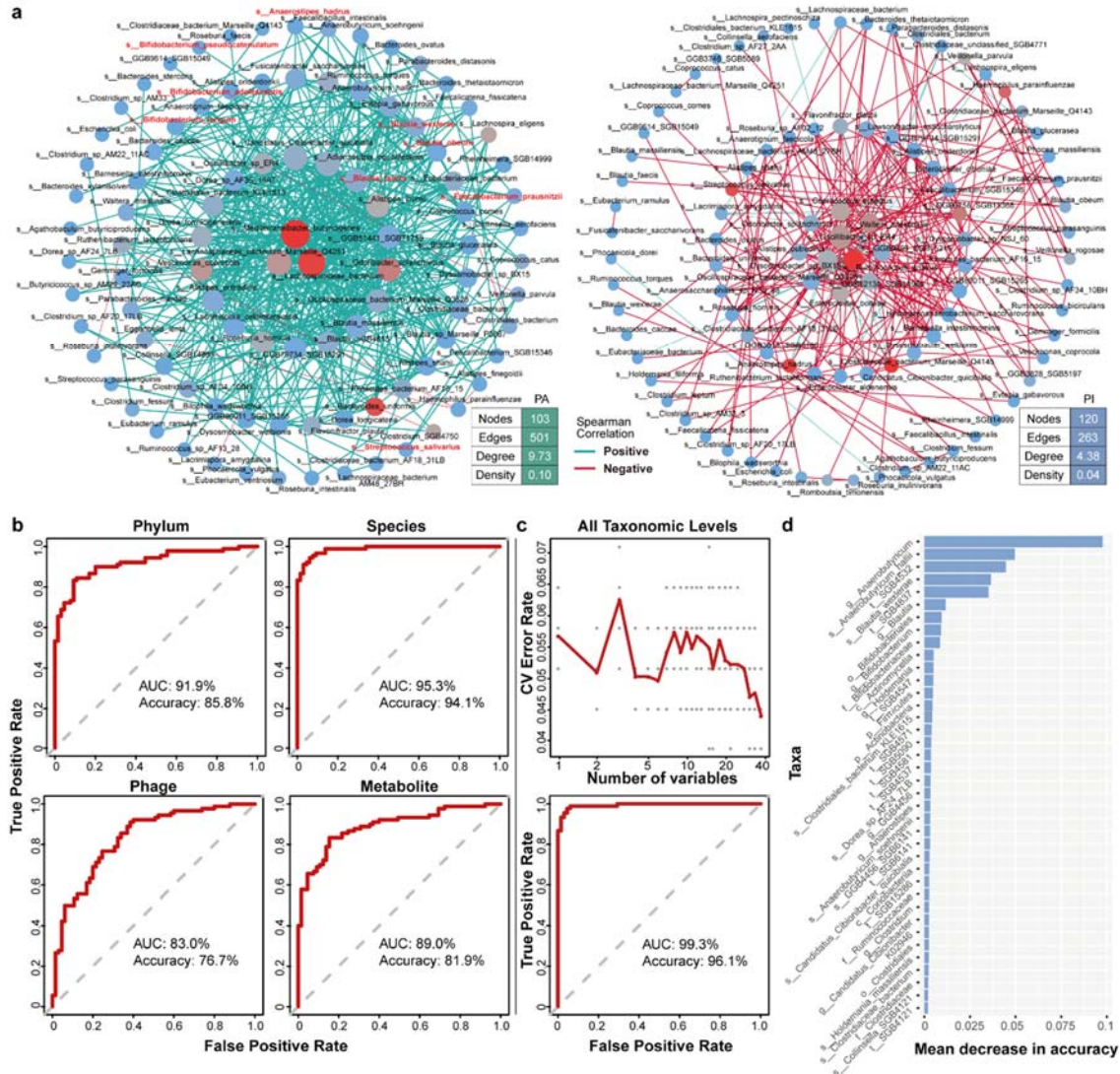


Figure 3. Ecological network organization and random forest (RF) classification distinguish PA and PI microbiomes.

(a) Species-level co-occurrence networks for PA (left) and PI (right), based on Spearman correlations of transformed relative abundances ($|r| \geq 0.4$, $P < 0.05$); edges represent significant positive (green) or negative (red) correlations. Node size is proportional to node degree. Insets summarize key metrics for each layer: number of nodes, edges, mean degree, and network density. **(b)** Receiver operating characteristic curves of the RF models trained on phylum, species, phage, and metabolite features. Performance assessed by area under the curve (AUC) and 10-fold cross-validation, with overall accuracy shown in each plot. The dashed diagonal line represents the performance of a random classifier (AUC = 0.5). **(c)** Cross-validated (CV) error rate of the all-taxonomic-level RF model as a function of top-ranked features retained. **(d)** Variable-importance (mean decrease in accuracy) for the final reduced model (38 most discriminatory taxa). This model achieved the highest AUC of 99.3%, with 96.1% accuracy.

These findings suggest significant structural and functional differences in the gut microbiota between the PA and PI groups, consistent with a modulatory role of habitual physical activity on the gut ecosystem. This includes altered interactions among microbial taxa, phages, metabolic pathways, and metabolites. Notably, the phage-related network differences are exploratory and should be interpreted as hypothesis-generating.

1.3.2.3 RF prediction models

To investigate the impact of regular physical activity on immunity, we developed RF prediction models, utilizing data from the gut microbiota and their functions. Initially, we constructed separate RF models using different levels of gut microbiota data, including the fecal phylum, species, phage, and KEGG pathway. These models achieved accuracies ranging from 76.7% to 94.1%, with corresponding area under the curve (AUC) values ranging from 83.0% to 95.3% (Fig. 3b). However, the performance of the RF model significantly improved when we focused on the top 38 species and functions that exhibited the highest discriminatory power across all classification levels. This refined RF model achieved an impressive accuracy of 96.1% in distinguishing between individuals that were PA and PI, with a ten-fold cross-validation AUC of 99.3% (Fig. 3c). The contribution of all 38 features to the overall accuracy of the RF model is illustrated in Fig. 3d.

This result underscores the effectiveness of the RF prediction model, which relies exclusively on gut microbiome data and achieves an accuracy rate as high as 96.13%. This model provides strong scientific support for using gut microbiome data to assess the impact of exercise on individual health outcomes.

1.3.3 Probiotic supplementation induced a partial shift toward an active-like microbiome in PA individuals

To determine whether probiotic supplementation can modulate the gut microbiome of insufficiently active individuals toward features observed in PA counterparts, we analyzed post-intervention samples from 90 PI participants randomized to receive either Probio-M9 (PIP, $n = 45$) or placebo (PIC, $n = 45$), comparing them to the PA reference group ($n = 65$) across taxonomic and functional levels (Fig. 4).

Figure 4

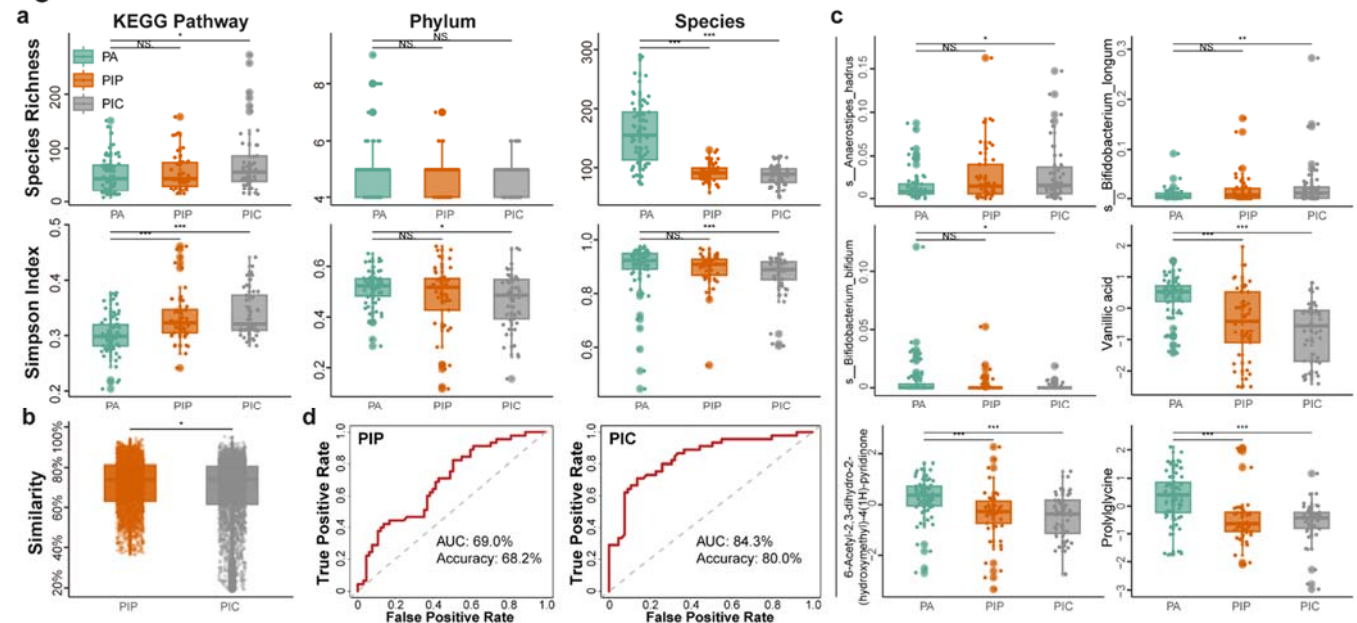


Figure 4. Probiotic supplementation induces a partial shift toward an active-like microbiome in insufficiently active individuals. (a) Alpha diversity (Chao1 richness, top; Simpson diversity, bottom) at different levels across PA, probiotic-supplemented PI participants (PIP), and placebo-treated PI participants (PIC). (b) Community similarity to PA, calculated as $1 - rJSD$ for all PIP-PA and PIC-PA comparisons. Higher values indicate greater resemblance to PA. (c) Relative abundances of key PA-associated species and levels of differential metabolites across PA, PIP, and PIC. (d) Receiver operating characteristic curves of RF models distinguishing PA from PIP (left) and PA from PIC (right) using multi-level microbiome features. The dashed diagonal line represents the performance of a random classifier (AUC = 0.5). Significance level (Wilcoxon rank-sum test): * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, $P > 0.05$.

At the phylum level, α -diversity (Simpson index) in PIP was comparable to PA ($P > 0.05$), whereas PIC remained significantly lower than PA (median = 0.486 vs 0.522; $P < 0.05$; Fig. 4a). Species richness (Chao1) did not differ among PA, PIP, and PIC ($P > 0.05$), consistent with baseline observations. At the species level, α -diversity in PIP likewise trended toward PA relative to PIC ($P > 0.05$), while species richness in both PIP and PIC was significantly lower than in PA (median: PA = 155, PIP = 91, PIC = 89; $P < 0.05$). For functional profiles, Simpson diversity was higher in both PIP and PIC than in PA, (median: PA = 0.298, PIP = 0.323, PIC = 0.321; $P < 0.05$), reflecting a pattern observed at baseline. In contrast, functional richness in PIP did not differ from PA ($P > 0.05$), whereas PIC remained significantly higher ($P < 0.05$).

To evaluate community-level shifts, we computed pairwise rJSD-based similarity (1 - rJSD) between each inactive participant and the PA group. Post-intervention, PIP showed greater similarity to PA (69.3%) than PIC (67.8%; $P < 0.001$; Fig. 4b), indicating a modest movement toward the PA microbiome configuration. As a complementary measure, we calculated centroid distances in rJSD-based PCoA space: the PIP-PA distance (0.307) was slightly smaller than PIC-PA (0.322), though this difference was not statistically significant, likely due to limited effect size over the 6-week intervention period.

Together, these results suggest that Probio-M9 supplementation induces a partial, measurable shift in the gut microbiome of insufficiently active individuals toward an active-like profile, without achieving full alignment with the PA group.

In the analysis of key taxa and metabolites (Fig. 4c), post-intervention levels of several species, *Anaerostipes hadrus*, *B. bifidum*, and *B. longum*, and metabolites, including vanillic acid, prolylglycine, and 6-Acetyl-2,3-dihydro-2-(hydroxymethyl)-4(1H)-pyridinone, in the PIP group became more closely aligned with those of the PA group ($P > 0.05$) while differing significantly from PIC ($P < 0.05$). As a major probiotic lineage in the human gut, *Bifidobacterium* is closely linked to barrier repair, anti-inflammatory and antioxidative responses, and may enhance tight-junction regeneration, mitochondrial biogenesis in muscle, and energy metabolism, thereby supporting exercise capacity and metabolic health^[52, 53]. Taken together, these findings suggest that probiotics may functionally remodel the intestinal ecosystem of inactive individuals by modulating core symbionts and their metabolic outputs.

We further evaluated group separability using a pre-established RF model (Fig. 4d). The classifier achieved an accuracy of 68.2% with an AUC of 69.0% for distinguishing PIP from PA, whereas it reached 80.0% accuracy with an AUC of 84.3% for distinguishing PIC from PA. The reduced separability between PIP and PA indicates a marked narrowing of their microbiome differences, while PIC remained more distinct. Overall, probiotic supplementation improved the gut microecological features of inactive individuals toward an active-like state, offering a potential strategy to mitigate sedentary-associated intestinal dysfunction.

1.4 Discussion

Previous studies have confirmed that regular physical activity can modulate the gut microbial community through multiple mechanisms, altering the composition and function of the gut microbiota^[7-9, 54].

Probiotic interventions have also been shown to influence the gut microecology in individuals who do not exercise regularly^[12, 55, 56]. However, it remains unclear whether probiotic supplementation in insufficiently active individuals can shift their gut microbiome toward features observed in regularly active counterparts. In this study, we first compared 65 PA and 90 PI young adults to characterize activity-associated differences in the gut microbiome and metabolome. We then analyzed data from a 6-week randomized, double-blind, placebo-controlled trial within the PI cohort, 45 participants received Probio-M9 and 45 received placebo, to assess whether supplementation induces movement toward an active-like microbial profile.

Cross-sectional analyses revealed notable differences between PA and PI groups across multiple layers of the gut ecosystem. The PA group exhibited significantly higher microbial α -diversity at the phylum level, consistent with prior reports linking physical activity to greater microbial richness and evenness^[45, 57, 58]. Differences were less pronounced at finer taxonomic levels, suggesting that exercise may primarily shape broad ecological properties rather than individual taxa. At the viral level, the PA group showed higher phage diversity but lower phage richness compared to PI, which may reflect altered bacteriophage-bacteria dynamics in active individuals^[1, 49, 58, 59]. Functionally, KEGG pathway-based diversity also differed between groups, indicating a reorganization of microbial functional potential associated with habitual activity.

These differences extended to β -diversity: PCoA and PERMANOVA confirmed significant divergence in community structure at the species, functional, and phage levels. This suggests that physical activity influences not only taxonomic composition but also the overall architecture of microbial interactions. Parallel metabolomic profiling revealed distinct fecal metabolite profiles between PA and PI, reinforcing the idea that exercise is linked to coordinated shifts in microbial metabolic output. Given that gut-derived metabolites mediate microbiota-host communication by influencing^[4-6, 60, 61], our findings support a model in which activity-associated microbial changes are accompanied by alterations in metabolic cross-talk along the microbiome-metabolite-host axis.

To identify specific microbial features associated with physical activity, we applied a dual analytical approach combining Wilcoxon rank-sum tests and MaAsLin2^[51]. The PA group showed higher relative abundance of Actinobacteria and lower Firmicutes compared to PI. Within this broad shift, several taxa were consistently enriched in PA, including *Faecalibacterium prausnitzii*, a major butyrate producer with anti-inflammatory and mucosa-protective properties^[62-65], and *Anaerostipes hadrus*, which converts lactate and acetate into butyrate and may link microbial fermentation to host energy metabolism^[66]. Multiple *Bifidobacterium* species, including *B. adolescentis*, *B. longum*, *B. pseudocatenulatum*, *B. bifidum*) were also elevated in PA and have been associated with barrier repair, redox balance, and immune maturation^[67-72]. Enrichment of select *Blautia* species (*B. wexlerae*, *B. faecis*, *B. obeum*) and *Streptococcus salivarius*, the latter linked to reduced upper respiratory infections^[73-77], further distinguished the PA profile. In contrast, potentially pro-inflammatory or metabolically adverse taxa, such as *Bacteroides fragilis* and *Eubacterium ventriosum*, were relatively depleted in PA^[78-81]. Rather than reflecting isolated changes, these shifts suggest

a coordinated reconfiguration of the gut community, from one enriched in taxa associated with inflammation or metabolic dysfunction toward a configuration dominated by symbionts involved in short-chain fatty acid (SCFA) production, barrier support, and immune regulation. This interpretation is supported by concomitant metabolite differences, though direct mechanistic links remain inferential.

At the viral level, our exploratory phageome analysis indicated enrichment of the family *Orlajensenviridae* in the PA group, raising the possibility that specific phage lineages may associate with exercise-related ecological states. However, without host linkage or functional validation, these observations should be considered hypothesis-generating. Similarly, while we identified distinct metabolites, such as higher 6-acetyl-2,3-dihydro-2-(hydroxymethyl)-4(1H)-pyridinone and lower steviobioside in PA, the biological relevance of these compounds requires further investigation. Taken together, the data indicate that habitual physical activity is associated with integrated changes across bacterial, viral, functional, and metabolite layers, even if the precise host consequences of many features remain unclear.

Co-occurrence network analysis provided additional insight into how physical activity reshapes gut ecological organization. At the species level, the PA group exhibited higher connectivity and network density, consistent with a cohesive bacterial backbone capable of distributing perturbations and supporting stable microbial guilds, including those involved in SCFA production. In contrast, the PI group showed a sparser species-level network but more complex and densely wired networks at the phage, KEGG pathway, and metabolite levels. This juxtaposition, a simplified taxonomic scaffold alongside intensified functional and phage-metabolite interactions, may characterize the gut ecosystem in insufficiently active individuals^[82]. One possible interpretation is functional buffering: a reduced set of bacterial hosts may rely on tightly coupled non-bacterial interactions to maintain metabolic output^[83]. However, such highly interconnected subnetworks could also be more vulnerable to cascading disruptions, consistent with reduced ecological resilience^[84]. Together, these patterns suggest that sedentary behavior is associated not only with shifts in taxonomic composition but also with a reconfiguration of interaction networks, moving from a robust, redundantly wired bacterial community in active individuals toward a functionally reconfigured, potentially fragile architecture in inactive individuals.

Random Forest models further highlighted the association between gut microbiome features and physical activity status. Using bacterial and functional markers, the model discriminated PA from PI individuals with high accuracy and cross-validated AUC. While this supports the potential of microbiome signatures as indicators of lifestyle patterns, their clinical utility requires external validation and careful control for confounders. Importantly, we interpret the RF performance as evidence of multivariate separation between groups, not as proof of causal effects of specific taxa on exercise capacity.

Against this cross-sectional backdrop, the probiotic intervention provides complementary evidence for the plasticity of the gut ecosystem in insufficiently active individuals. In the 6-week randomized, double-blind, placebo-controlled trial, Probio-M9 supplementation was associated with a partial shift of the PIP microbiome toward features observed in the PA group across diversity, community structure, and

functional traits. At the diversity level, Simpson's index in PIP no longer differed from PA, whereas PIC remained significantly lower, suggesting that Probio-M9 may modestly improve community evenness, an effect consistent with prior reports of probiotic-mediated microecological modulation^[85, 86].

At the community-structure level, rJSD-based similarity between PIP and PA was higher than between PIC and PA, indicating a small but measurable movement toward an active-like configuration. This shift was not driven by isolated changes in the supplemented strain: reads from *Lacticaseibacillus rhamnosus* were excluded from β -diversity and RF analyses to avoid artifactual inflation of similarity. Centroid distances in ordination space showed a consistent directional trend, though the difference was not statistically significant, likely reflecting the modest effect size over a short intervention window. These findings are consistent with a broad ecological response, possibly involving niche competition or community-level interactions that may suppress opportunistic taxa and promote stability^[87, 88]. Functionally, species richness in PIP did not differ from PA, unlike PIC, suggesting that the intervention may influence metabolic potential through pathway-level adjustments rather than changes in taxon count. Notably, several taxa and metabolites that shifted toward PA levels in PIP overlapped with those enriched in the cross-sectional PA profile, including *Anaerostipes hadrus*, *Bifidobacterium bifidum*, *B. longum*, vanillic acid, and prolylglycine. Given that *Lacticaseibacillus rhamnosus* produces lactate and *Anaerostipes hadrus* can convert lactate to butyrate^[54, 66, 89], these parallel changes are consistent with a hypothesized lactate-to-butyrate cross-feeding axis. However, without direct measurements of SCFAs or host inflammatory markers, this remains a testable hypothesis, not demonstrated mechanism.

Post-intervention RF analyses further supported this interpretation: PIP was less separable from PA than PIC (AUC 0.690 vs. 0.843), reflecting reduced multivariate distance. Together, these findings suggest that Probio-M9 may promote modest, ecologically coherent shifts along axes that distinguish PA from PI microbiomes, particularly those related to SCFA-linked taxa, barrier-associated functions, and immunometabolic pathways. Nevertheless, causal inference is limited. The observed changes are hypothesis-generating and may reflect a combination of niche competition, metabolic cross-feeding, or indirect host-mediated immune effects. Future studies with targeted metabolomics and host phenotyping are needed to validate these mechanisms. Importantly, the probiotic-induced changes represent partial ecological and functional alignment, not equivalence, to the active state. Exercise confers broad physiological benefits that extend far beyond the gut microbiome; probiotics should not be interpreted as a substitute.

Overall, our findings support a working model in which regular physical activity fosters a resilient, interconnected gut ecosystem, while probiotic supplementation in insufficiently active individuals can induce a partial shift along overlapping microbial and metabolic axes. From a translational perspective, this suggests probiotics may serve as a practical adjunct for those unable to increase physical activity in the short term, complementing, not replacing, lifestyle interventions.

Several limitations warrant acknowledgment. First, physical activity was assessed via self-report, introducing potential misclassification; objective measures (e.g., accelerometry) were not used. Second, although participants were drawn from a relatively homogeneous university cohort, detailed dietary intake was not quantified, leaving residual confounding possible. Third, the 6-week intervention lacked post-supplementation follow-up, limiting conclusions about the durability of effects, microbiome changes may revert after cessation. Finally, the absence of host phenotyping (e.g., inflammation, barrier markers, fitness metrics) prevents direct links between microbial shifts and clinical outcomes. Future studies in larger, more diverse populations, with objective activity monitoring, standardized dietary and lifestyle assessments, longitudinal sampling, and integrated host-microbe profiling, will be essential to evaluate the generalizability, stability, and health relevance of microbiome shifts toward active-like profiles.

1.5 Conclusions

This study demonstrates that regular physical activity and insufficient activity are associated with distinct gut microbial and metabolic profiles in young adults. Physically active individuals exhibited higher α -diversity, a more cohesive community structure, and enrichment of commensal taxa such as *Bifidobacterium* and *Anaerostipes hadrus*, alongside metabolites linked to barrier integrity and anti-inflammatory function. In insufficiently active participants, a 6-week randomized, double-blind, placebo-controlled trial found that supplementation with Probio-M9 was associated with a modest shift toward an active-like microbiome configuration. This was evidenced by increased rJSD-based similarity to the PA group, movement of specific taxa and metabolites toward PA levels, and reduced RF separability between PIP and PA compared with PIC and PA.

These changes were partial in magnitude and should be interpreted in light of several limitations: physical activity status was based on self-report, detailed dietary intake was not quantified, and the intervention duration was short without post-cessation follow-up, factors that may affect generalizability and durability. Importantly, probiotic supplementation should be viewed as a complementary, microbiota-targeted approach for individuals unable to increase activity in the short term, not as a substitute for exercise or its systemic health benefits. Future studies incorporating objective activity monitoring, standardized dietary assessment, longer intervention periods with follow-up, and integrated host-microbiome phenotyping are needed to assess the stability, mechanisms and clinical relevance of probiotic-induced shifts along the microbiome-metabolite-host axis, and to inform the development of individualized, scalable microbiome-based strategies.

Ethical approval

The study design and protocols were approved by the Ethics Committee of the Inner Mongolia Nutrition Society (A202207019), adhering to rigorous ethical standards. All participants provided written informed consent prior to their involvement in this study.

Consent for publication

Not applicable.

Competing interests

The author(s) report no potential conflicts of interest.

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Availability of data and materials

Raw metagenomic sequencing data are available in the GSA Sequence Read Archive at <https://ngdc.cncb.ac.cn> under accession ID CRA018709 (accessible at <https://ngdc.cncb.ac.cn/gsa/s/H61Y5vZl>).

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