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RESEARCH ARTICLE

Exercise-changed gut mycobiome as a potential contributor to metabolic benefits in diabetes prevention: an integrative multi-omics study

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ABSTRACT

Background: The importance of gut microbes in mediating the benefits of lifestyle intervention is increasingly recognized. However, compared to the bacterial microbiome, the role of intestinal fungi in exercise remains elusive. With our established randomized controlled trial of exercise intervention in Chinese males with prediabetes (*n* = 39, ClinicalTrials.gov:NCT03240978), we investigated the dynamics of human gut mycobiome and further interrogated their associations with exercise-elicited outcomes using multi-omics approaches.

Methods: Clinical variations and biological samples were collected before and after training. Fecal fungal composition was analyzed using the internal transcribed spacer 2 (ITS2) sequencing and integrated with paired shotgun metagenomics, untargeted metabolomics, and Olink proteomics.

Results: Twelve weeks of exercise training profoundly promoted fungal ecological diversity and intrakingdom connection. We further identified exercise-responsive genera with potential metabolic benefits, including Verticillium, Sarocladium, and Ceratocystis. Using multi-omics approaches, we elucidated comprehensive associations between changes in gut mycobiome and exercise-shaped metabolic phenotypes, bacterial microbiome, and circulating metabolomics and proteomics profiles. Furthermore, a machine-learning algorithm built using baseline microbial signatures and clinical characteristics predicted exercise responsiveness in improvements of insulin sensitivity, with an area under the receiver operating characteristic (AUROC) of 0.91 (95% CI: 0.85–0.97) in the discovery cohort and of 0.79 (95% CI: 0.74–0.86) in the independent validation cohort (*n* = 30).

Conclusions: Our findings suggest that intense exercise training significantly remodels the human fungal microbiome composition. Changes in gut fungal composition are associated with the metabolic benefits of exercise, indicating gut mycobiome is a possible molecular transducer of exercise. Moreover, baseline gut fungal signatures predict exercise responsiveness for diabetes prevention, highlighting that targeting the gut mycobiome emerges as a prospective strategy in tailoring personalized training for diabetes prevention.

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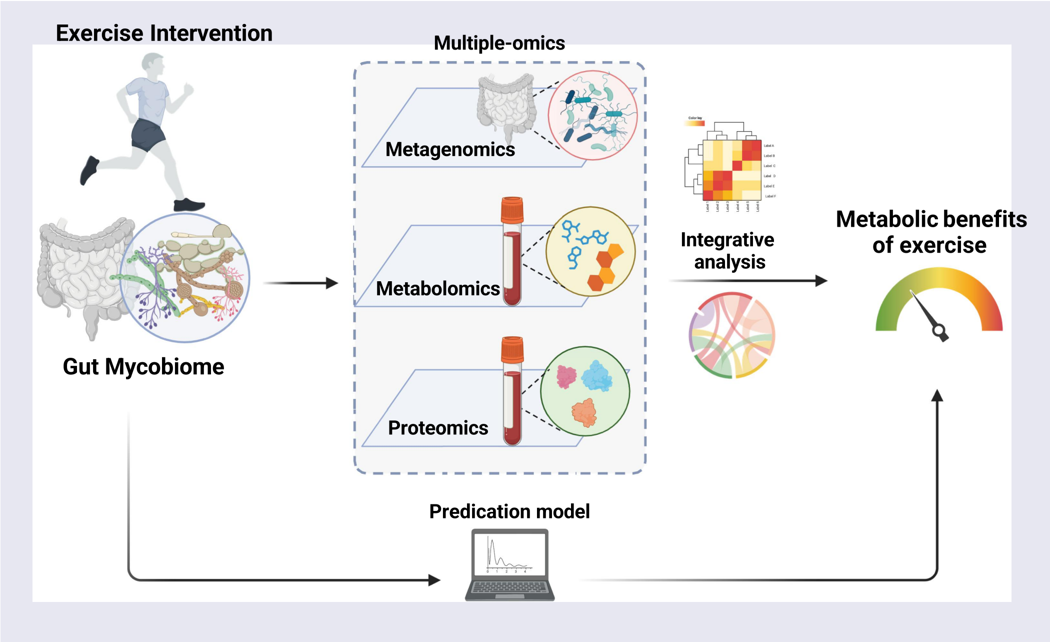

Introduction

Intestinal microbes are pivotal in orchestrating host immunologic and metabolic homeostasis.[1] Alongside bacterial microbiomes, fungal communities (mycobiome) also colonize the human gastrointestinal tract.[2] Though it only constitutes 0.1% of the entire gut ecosystem,[3] emerging evidence demonstrated its importance in host health. Gut fungal dysbiosis is associated with the onset or progression of various bowel diseases and colorectal cancer.[4] In metabolic contexts, human gut mycobiome composition is related to the progress of nonalcoholic fatty liver disease.[5] *Mucor* genus and *Candida spp*. are predominated in obese subjects and diabetic patients compared to their healthy counterparts, respectively.[6,7] Moreover, *Candida parapsilosis* and *Candida albicans* transplantation evoked metabolism syndromes in mice models.[8,9]

Physical exercise is an effective non-pharmacological approach to diabetes management with pleiotropic benefits.[10] A previous study has demonstrated distinct patterns of commensal bacteria between athletes and individuals with sedentary lifestyles.[11] Furthermore, the fermentation and composition of the bacterial microbiome are dramatically remodeled by exercise and determine differential exercise responsiveness in overweight individuals with prediabetes.[10] Yet, compared to gut bacteria, the impact of exercise on the gut mycobiome and the potential contribution of intestinal fungi in exercise physiology remain obscure.

Accumulating evidence has shown that the gut mycobiome is resilient to habitual lifestyles. For example, fecal *Candida* abundance positively correlates with dietary carbohydrate consumption in humans.[12] Likewise, low-calorie diet intervention could effectively restore the *Mucor* abundance in obese patients.[13] Murine studies showed that the gut mycobiome was predominated by *Fusarium* and *Alternaria* with a standard chow diet, whereas *C. albicans* was the most abundant fungi upon treatment with an obesogenic diet.[14] Thus, we reasoned whether exercise remodels enteric fungal profiles, which in turn contributes to the metabolic benefits conferred by exercise.

Herein, we investigated the dynamics of the human gut mycobiome in a well-designed randomized controlled exercise trial on medication-naive overweight males.[10] We further interrogated the relationship of exercise-shaped gut mycobiome with bacteriome, metabolome, and proteome in modulating the metabolic benefits of exercise. Moreover, we developed a machine-learning algorithm integrating baseline fungal signatures which can predict the individual responsiveness to exercise intervention in diabetes prevention.

Materials and methods

Study design

The study was approved by the ethics committees of the Hospital Authority Hong Kong West Cluster (UW15–370) and registered on ClinicalTrials.gov (NCT03240978). Thirty-nine overweight males with prediabetes were enrolled for a randomized clinical trial (RCT) of 12-week supervised exercise training as described.[10] In brief, overweight (body mass index (BMI) > 25 kg/m^2) Chinese males aged 20 to 60 years old with prediabetes (defined as impaired fasting glucose [5.6 mmol/L to 6.9 mmol/L] and/or impaired glucose tolerance [2-h blood glucose level of oral glucose tolerance test (OGTT): 7.8 mmol/L to 11.0 mmol/L after a 75-g oral glucose challenge]). Moreover, all volunteers are on medication-naïve and in the absence of any chronic diseases or mental illness and lack regular exercise training. Eligible subjects who signed written informed consent were randomly assigned to exercise ($n = 20$) or sedentary groups ($n = 19$) to undergo supervised high-intensity interval exercise training (3 sessions/week, for 12 weeks) (Supplementary Table S9) which was designed referred to prior study.[15,16] This exercise protocol combined both aerobic and strength training, which has been recognized for its effectiveness in improving insulin sensitivity.[17] The details of our training regimen were elaborated in our previous publication.[10] Briefly, volunteers received a warm-up exercise (10 min), a high-intensity interval training (about 50 min with 80–95% of the maximal heart rate), together with a stretching and cool-down training. At least 85% attendance of exercise was required for data analysis. Exercise responsiveness was evaluated accordingly to interpersonal variability in terms of improvement in the insulin resistance index (HOMA-IR). Exercise non-responders were determined as subjects who failed to ameliorate HOMA-IR greater than the 2-fold technical error and *vice versa* according to previous publications.[10,18] Another 30 subjects were recruited as a validation cohort with the same inclusion criteria and exercise training regimen.

Sample collection and clinical data measurement

All participants were subjected to monthly specimen collection throughout the 12-week exercise training program. After overnight fasting (about 10–12 hours), blood samples were collected, aliquoted, and stored at −80°C for further analysis. Serum glucose level and lipids profiles, in terms of triglycerides (TG), total cholesterol (TC), high-density lipoprotein (HDL) cholesterol, and low-density lipoprotein (LDL) cholesterol, were measured by laboratory biochemistry analyzer (Hitachi 717, Roche Diagnostics, Germany) following instruction. Insulin level was assayed by commercial ELISA kits (Immunodiagnostics, Hong Kong SAR). HOMA-IR was calculated as fasting insulin × fasting glucose/22.5, whereas the Matsuda index was calculated using the result of OGTT as 1000/[fasting glucose × fasting insulin × mean glucose (during OGTT) × mean insulin levels (during OGTT)]$^{1/2}$. Body composition and exercise performance including strength, flexibility, and maximal oxygen consumption were determined before and after exercise following standardized protocols as described.

Circulating proteomics and targeted metabolomics signatures

Circulating proteomics was detected by Olink Proteomics (Uppsala, Sweden) with Olink Explore cardiometabolic and inflammation panels.[19] Specifically, protein biomarkers were identified using the Olink Explore 384 panels for cardiometabolic and inflammation. In brief, the proximity extension assay (PEA) technique employs DNA oligonucleotide-tagged antibody pairs to bind to target proteins. When two compatible antibodies attach to the target protein, the oligonucleotide pairs combine and are lengthened by DNA polymerase, forming a distinct DNA barcode that is later analyzed using next-generation sequencing. Due to the requirement of properly matched DNA string pairs for the generation of detectable and quantifiable signals, the PEA technique demonstrated high specificity and outstanding sensitivity.[20] The values were shown as normalized protein expression (NPX) units on a log2 scale. Targeted metabolomics profiling of human plasma samples was determined using gas or liquid chromatography coupled to mass spectrometers by Metabo-Profile (Shanghai, China).[10]

Fecal DNA extraction and sequencing

We extracted fecal DNA from freezing stool samples using PowerFecal Pro DNA Kit (QIAamp, QIAGEN, Germany). Internal transcribed spacer 2 (primers: sense 5'-GCATCGATGAAGAAC GCAGC-3' and antisense 5'-TCC TCCGCTTATTGATATGC-3') sequencing was conducted using NovaSeq PE250 with a data depth of 100K tags per sample (Novogene, Tianjin, China). Matched metagenomics data were used in our published dataset (NCBI Sequencing Read Archive under BioProject ID PRJNA454826) and re-analyzed by Metaphlan 3.0 and HUMAnN 3.0.[21,22]

ITS2 and metagenomics analysis

Human reads contamination, PCR adapter, low-quality reads, and duplicated reads of raw metagenomic shotgun sequencing were removed with a described pipeline.[23] Quality control of raw ITS2 sequencing reads was performed by filtering and trimming the adapter sequences, primers, and poly-A tails with cutadapt.[24] The relative abundance of the microbial community from the metagenome was calculated with MetaPhlAn3 at different taxonomic levels. PIPITS pipeline was used for ITS data with default settings including further quality filtering, read-pair merging, ITS2 filtering, and chimera removal.[25] The remaining reads were further binned based on 97% similarity as an operational taxonomic unit (OTU) and aligned to the UNITE fungal database (Version 9.0) with the Mothur classifier.[26]

Fungal and bacterial abundance

The abundance of fungi from ITS sequencing data annotated by PIPITS was further normalized by the relative abundance. The relative abundance of bacteria from metagenomic data was retrieved from MetaPhlAn3 at each taxonomic level as previously described. The taxonomic variation of fungus or bacteria at each level before and after exercise was retrieved from the fold change of fungal relative abundance of individuals. HUMAnN3 was used with metagenomic reads to estimate gene family abundances. Reads per kilobase (RPK) values for gene family abundances were copies per million (CPM) normalized. KEGG pathways were further annotated.

Diversity analysis

The alpha diversities in Simpson, Shannon, and Chao1 indexes of the bacterial and fungal communities were calculated using the vegan package.[27] The Bray-Curtis dissimilarity of the fungal species among individuals was performed by the Phyloseq package.[28]

Differential analysis

To identify the significantly different fungus within subjects before and after exercise, MaAsLin2[29] was implemented with the paired compound Poisson linear model (CPLM) by normalizing the fungal relative abundance in three different ways: TSS, TMM, and CSS. The fungus with an adjusted p-value of <0.25 in at least two of the three normalizations was regarded as the significant result. The relative abundance of bacteria was compared within subjects between the two time points with the Wilcoxon signed-rank test for paired data and adjusted with Benjamini–Hochberg correction for multiple comparisons. Moreover, to evaluate the potential impact of the bacterial shift on shaping fungal abundance, MaAsLin2 was further used for testing the difference of identified significant fungal genera with the adjustment for all significantly changed bacteria genera (Supplementary Table S10).

Metabolic pathway enrichment analysis

KEGG Pathway enrichment analysis with significantly different metabolites was performed with MetaboAnalyst.[30] KEGG Pathway enrichment analysis with significantly different OLINK proteomic biomarkers was conducted with the clusterProfiler package.[31]

Machine learning model construction

Baseline fungal abundance at phylum, family, and genus level, the clinical parameters, and the bacterial abundance were compared between responders and non-responders with the Wilcoxon rank-sum test, and differentially abundant features with $p < 0.1$ were selected for constructing the machine learning models. Random forest models for classifying responders and non-responders were constructed in three ways: baseline mycobiome and clinical parameters, baseline microbiome and clinical parameters, and the combination of the three profiles with the Caret R package.[32] The model was trained with *ranger* function in our discovery cohort, using 10-repeated 10-fold cross-validation (repeatedcv) as a resampling method and ROSE sampling strategy to account for the imbalance in the two classes.[33] The constructed model was further tested in an independent validation cohort as previously described.[10] To perform a robust and generalizable analysis, the machine learning algorithm was iterated 100 times in both discovery and validation cohorts. Model performance was assessed using the evalm function from the Mleval R package, which included the sensitivity, specificity, the Matthews correlation coefficient (MCC), and net present value (NPV).

The area under the curve (AUC) of the receiver operating characteristic curve (ROC) curve was calculated by evalm function and further visualized with the pROC R package. The AUCs of each model were used as the main indicator of model performance and were further compared with DeLong's test, using the roc.test function from the pROC R package. Results with a *P*-value of <0.05 were considered statistically significant.

Mediation analysis

To investigate whether the presence of important fungal species at baseline may affect the change of phenotypes via the baseline level of OLINK, metabolites, and microbial pathway, we implemented bi-directional mediation analysis using the mediate package in R. In detail, we estimated the total effect from the presence (represented in 0 or 1) of fungal at baseline to the fold change of phenotypes after exercise, the effect on the baseline abundance of the potential mediators, and the effect of the baseline abundance of the mediators to the fold change of phenotypes after exercise. We further performed the causal mediation analysis among the three models. We then selected the significant results of all the effects among the three models as well as the indirect effect. Lastly, we filtered the significant results of the direct effect to generate the final result.

Data visualization

All the box plots, bar charts, and dot plots were constructed with ggplot2 unless otherwise stated.[34] Sankey plots were built with the NetworkD3 package.[35] Networks were visualized with Cytoscape.[36] Heatmaps were constructed with the ComplexHeatmap package.[37] The phylogenetic tree was visualized with *ggtree* package.[38] The AUROC plot was built with the PROC package.[39]

Statistical analysis

All statistical analyses were performed in R software. The group comparison of alpha diversity and fungal abundance between samples before and after exercise was conducted with the Wilcoxon signed-rank test. Adaptive false discovery rate (FDR) correction was applied for multiple comparisons of the differential abundance analysis. Principal components analysis (PCA) of the fold change of fungal species was performed with the *vegan* package. Multivariate analysis PERMANOVA was performed using *vegan* for 1000 permutations. The *envfit* function of *vegan* package was used to analyze the relationship between relative changes in the clinical parameters and their overall fungal composition with 1×10^4 permutations. The group dispersion between exercise and control was calculated by *vegan* and was further evaluated with ANOVA. The co-abundance network analysis using pairwise Spearman correlations between fungi before and after exercise. The Spearman correlation was performed between the fold change of fungal abundances and multi-omics including clinical parameters, metabolomics, and proteomics. Benjamini–Hochberg procedure with the cutoffs of 0.25 and 0.05 was applied to all the results. Bi-directional mediation analysis was performed with the *mediation* package, which was constructed with the presence/absence of fungi at baseline, the baseline abundance of metabolites and proteomics, and the fold change of host phenotypes. Machine learning built by a random forest model was implemented with the *caret* package.

Results

Exercise training remodels gut mycobiome composition

To illustrate the impact of exercise training on gut mycobiome composition, fecal samples from exercise intervention and sedentary control groups were collected for ITS2 sequencing. We annotated 9 phyla, 187 families, 258 genera, and 253 species with >10% prevalence among all fecal samples (Supplemental Table S1). The *Ascomycota* phylum predominated 85.73% of the gut mycobiome, while *Saccharomyces* was the most enriched genus with a mean relative abundance of 39.08% (Supplemental Figure S1). As a major confounding factor, the dietary intake was evaluated, and no significant difference in nutrition was found between the two groups (Supplemental Figure S2).

Notably, the exercise resulted in a significantly increased fungal α-diversity, which was unchanged in the control group ($p = 0.38$) (Figure 1(a, b)). Consistently, the microbial change within subjects measured by the Bray-Curtis distance in the exercise group was significantly higher than in the sedentary group at genus levels (Figure 1(c)). Moreover, the exercise effectively remodeled both the centroid and dispersion of the fungal community compared to their baseline (FDR < 0.05), whereas no obvious change was found within the control subjects (Figure 1(d), Supplemental Table S2). The majority of fungal genera (82.52%) remained stable during exercise without significant alternation. By contrast, 17.02% of them showed increased abundance levels and only 2.13% decreased (FDR < 0.05). (Supplemental Figure S3 and Supplemental Table S3). Despite the personalized dynamics in responding to exercise training at the genus level (Figure 1(e) and Supplemental Figure S4), a group of exercise-responsive fungal genera was identified (Figure 1(f) and Supplemental Figure S5). The relative abundance of *Verticillium* exhibited a 1.5-fold increase from baseline after training, distinguished from sedentary groups. Relative abundances of other genera, including *Chloridium*, *Iodophanus*, *Monosporascus*, *Beauveria*, *Ceratocystis*, and *Bipolaris*, were augmented by over 30% after training and were significantly different compared to sedentary controls (Figure 1(f)). Interestingly, the abundance of *Verticillium* was notably decreased in children with clinical type-1 diabetes, and *Ceratocystis* level was higher in healthy subjects and associated with euglycemia status.[13,40] Therefore, the shifts in fungal abundance observed following exercise in our study align with previous clinical studies regarding their association with host glucose metabolism.

Physical exercise has been shown to modulate the gut microbiome, with one of the effects being the enhancement of bacterial community interactions.[10] Similar responses in gut fungi were found by co-abundance analysis (Figure 1(g)). At baseline, only 37 correlations within the fungal ecosystem were identified, whereas the interactions drastically surged to 80 after training. Importantly, the nexus is centralized around exercise-responsive fungi, like *Verticillium*, *Iodophanus*, *Monosporascus*, *Bipolaris*, and *Conocybe*. Our data implied that exercise-promoted fungi could be instrumental in enhancing fungal ecosystem communication. These findings collectively demonstrated exercise as a potent intervention in shaping gut mycobiome composition.

Alterations in gut mycobiome composition are associated with exercise-improved clinical parameters

Next, we performed a Spearman correlation analysis to determine the relationships between shifts in fungal abundances and improvements in metabolic phenotypes after exercise. The influence of host factors on fungal compositional alterations was assessed as measured by the Bray-Curtis distance. Multiple regressions of clinical parameters with permutation test revealed that fasting glucose level was associated with overall gut mycobiome variations with significant power ($R^2 = 0.35$; $p < 0.05$) (Figure 2(a) and Supplementary Table S5), which was consistent with prior reports about the bidirectional influences between the gut microbiome and host glucose metabolism.[41]

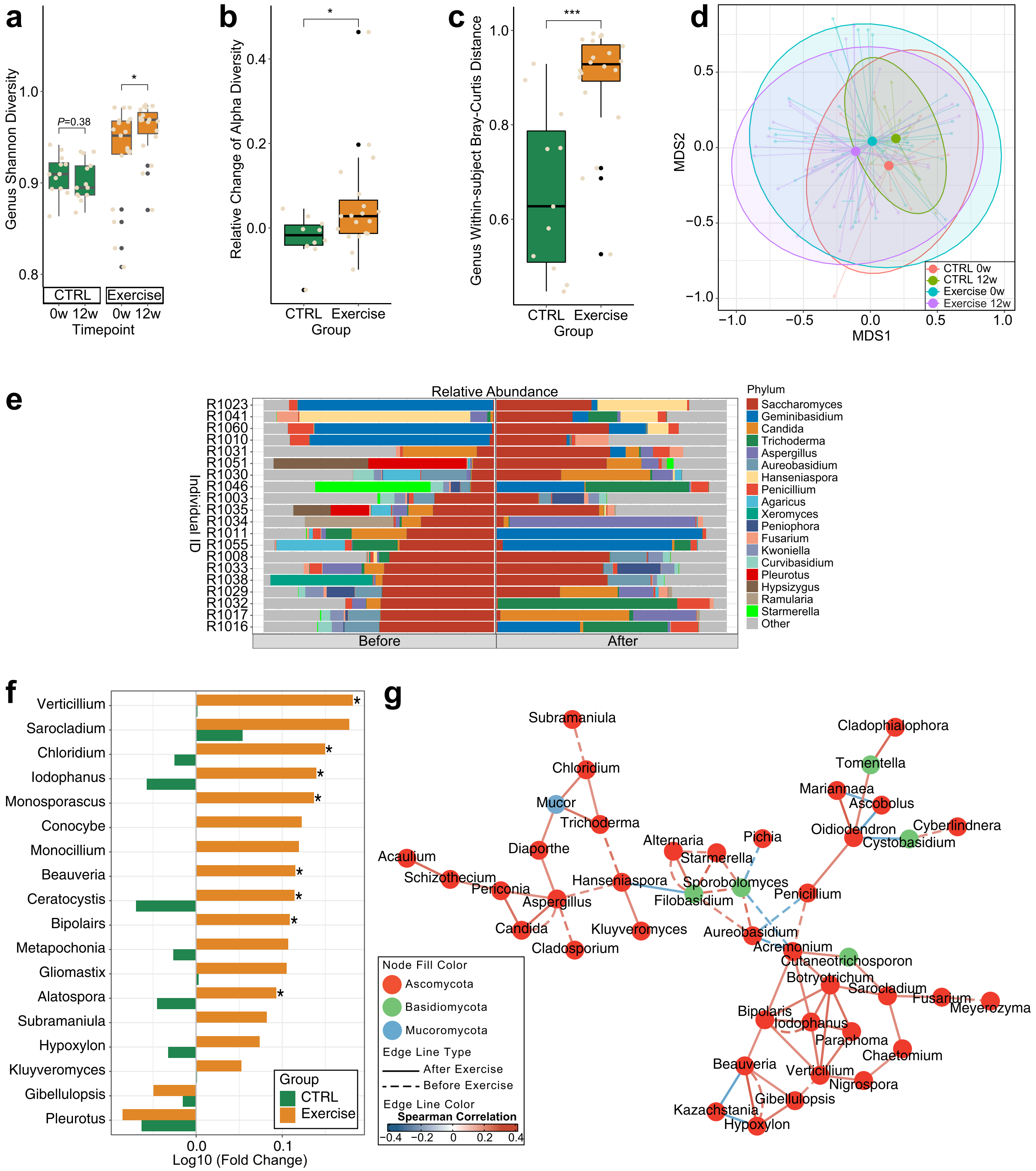

Figure 1. Physical exercise training reshapes gut fungal composition. Exercise training significantly remodeled overall fungal compositions at the genus level as evidenced in (a) alpha-diversity in the exercise group (Exercise, $n = 20$) and sedentary control group (CTRL, $n = 19$) measured by Shannon index; (b) changes of alpha-diversity within each subject, and (c) within-subject beta-diversity (measured by Bray-Curtis dissimilarity) between 0-week and 12-week in two groups. (d) NMDS plot of log2 fold change of genus abundance within subjects during exercise. The confidence ellipses (level = 0.95) were constructed in the exercise group at baseline (blue), after training (purple); and the control group at baseline (red) and after 12 weeks of exercise intervention (green). (e) The relative abundance of fungal phyla in individuals before and after exercise training. (f) The mean value of the fold change of significantly altered fungal genus composition (log10 transformed) in both exercise and control subjects. The fold change was divided by fungal relative abundance at 12 weeks to 0 weeks for each individual. (g) Exercise intervention promoted fungal communication as showed by co-abundance network analysis. The edges indicate statistically significant ($p < 0.05$) Spearman correlations between species present in at least 50% of subjects. The nodes are colored based on their affiliated phyla. Dashed and solid lines represent correlations before and after exercise, respectively. Lines in orange and blue represent positive and negative correlations.

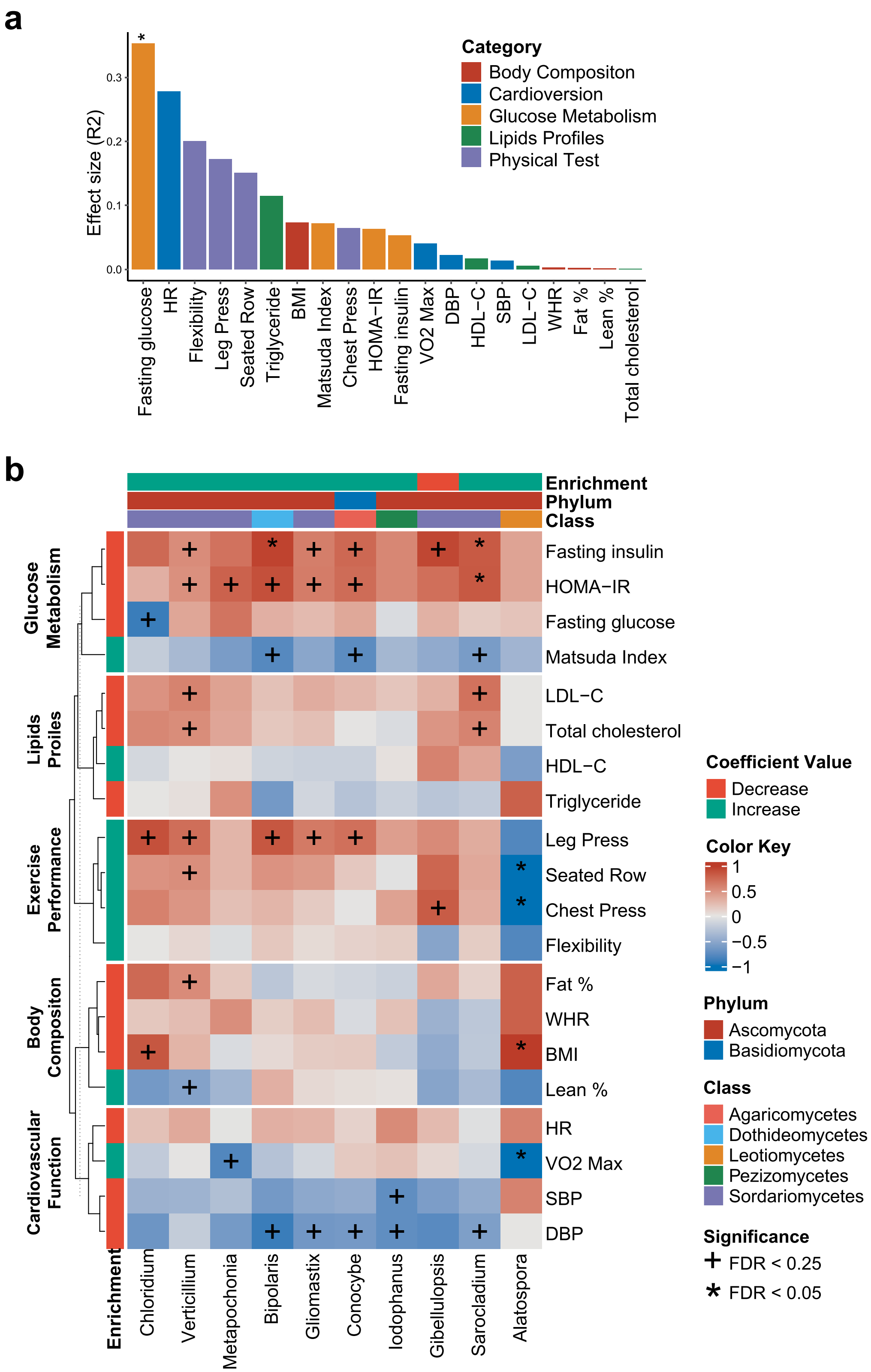

Figure 2. The taxonomic alterations of gut mycobiome are tightly associated with exercise-induced improvement in metabolic health. (a) The influence of host factors on human gut mycobiome at the Bray-Curtis distance was evaluated by permutational multivariate analysis of variance (PERMANOVA, permutation = 999). The bars were colored according to metadata categories. (b) Spearman correlation between the fold changes in significant fungal genus and fold changes in metabolic health after exercise training ($n = 20$). Significant results (FDR < 0.25) were marked with a plus symbol. *FDR < 0.05, $^{+}$FDR <0.25.

The increases in fungal abundance, including *Verticillium*, *Gliomastix, Conocybe* (FDR < 0.25), *Bipolaris*, and *Sarocladium* (FDR < 0.05) after exercise were positively associated with improvements in host insulin sensitivity, as indicated by fasting insulin level and HOMA-IR. In terms of lipids profiles, reductions in LDL and cholesterol contents were concomitant with the augment of *Verticillium* and *Sarocladium* after exercise (FDR < 0.25) (Figure 2(b)). By contrast, no correlations were detected between the mycobiome and HDL or triglyceride levels. Notably, increases in *Verticillium* were parallelly associated with the gain of leg and back strength, suggesting that changes in *Verticillium* abundance may be related to exercise performance (Figure 2(b)). In addition, its elevation was also positively associated with the reduction in fat mass percentage (Fat%). We further observed improvements in diastolic blood pressure (DBP) were remarkably linked with shifts in the gut fungal genera, with *Bipolaris*, *Gliomastix, Conocybe, Iodophanus*, and *Sarocladium* enclosed (Figure 2(b)). Collectively, our results suggested fluctuations in the gut mycobiome are tightly associated with exercise-elicited metabolically beneficial effects.

Trans-kingdom crosstalk between gut fungal and bacterial microbiome during exercise

Microbial synergism between the intestinal fungal and bacterial microbiome profoundly remodels the gut environment and holistic immunity.[42] We then probed into potential gut fungi-bacteria crosstalk during the exercise intervention.

There was a significant association in the shifts of α-diversity between fungi and bacteria at genus levels ($R^2 = 0.33$, $p = 0.04$), indicating a possible coordinated response of these two communities adapting to exercise (Figure 3(a)). We next assessed the changes in their interkingdom communication after training. Among those linkages, changes in *Verticillium* and *Sarocladium* showed prominent associations with exercise-shifted bacterial species (Figure 3(b)). Interestingly, *Veillonella* is an exercise-responsive bacterium enriched in elite marathon athletes, which metabolizes lactic acid into propionate to improve exercise performance.[43] Decreases in *V. infantium* were negatively associated with changes in exercise-promoted fungal species, especially with *Verticillium*, *Sarocladium*, and *Monocillium* (FDR < 0.25). Other bacteria typically enriched in non-obese or non-diabetic healthy controls, such as *Haemophilus parainfluenzae* and *Bifidobacterium dentium*,[44,45] also displayed negative correlations with the gain of *Monocillium* and *Gliomastix* (FDR < 0.25). In contrast, bacteria linked to metabolic syndrome, including *Clostridium symbiosum* (enriched in T2D), *Fusicatenibacter saccharivorans* (enriched in fatty liver disease), and *Dorea formicigenerans* (enriched in obesity),[45–47] showed opposite trends with proliferations in exercise-induced fungi. Hence, our results indicated a possible interaction between gut fungi and bacteria in response to exercise.

Further, by examining the correlation between gut fungi and bacterial function retrieved from the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologues (KOs), we observed that *Kluyveromyces* exhibited over 100 interactions with bacterial functions (Figure 3(c)). Of interest, *Kluyveromyces* was significantly elevated by 12.9% after training (Figure 1(e)). Lines of evidence also suggest that *Kluyveromyces* is a probiotic candidate with multiple metabolic benefits.[48] Mapping the fungi-related KOs into the KEGG Module level revealed that changes in the gut mycobiome had significant associations with histidine biosynthesis (Figure 3(d)), a potential mediator that bridges gut fungi with host cholesterol metabolism and the development of nonalcoholic fatty liver disease.[49,50] Interestingly, this finding was further substantiated by our metabolomics profiles that circulating histidine concentration was significantly increased after exercise training (supplemental Figure S6). Moreover, annotating the significant KOs at pathway levels highlighted that fungi were most related to metabolic pathways followed by the biosynthesis of secondary metabolites and cofactors (Figure 3(e)). In summary, we showed that exercise-induced gut fungi perturbations were accompanied by both compositional and functional transitions of gut bacteria.

Exercise-shaped changes in gut fungal composition correlate closely with alterations of host circulating metabolites and proteins

Fungi-produced metabolites potently impact host metabolism and immunity.[51] Pathway enrichment

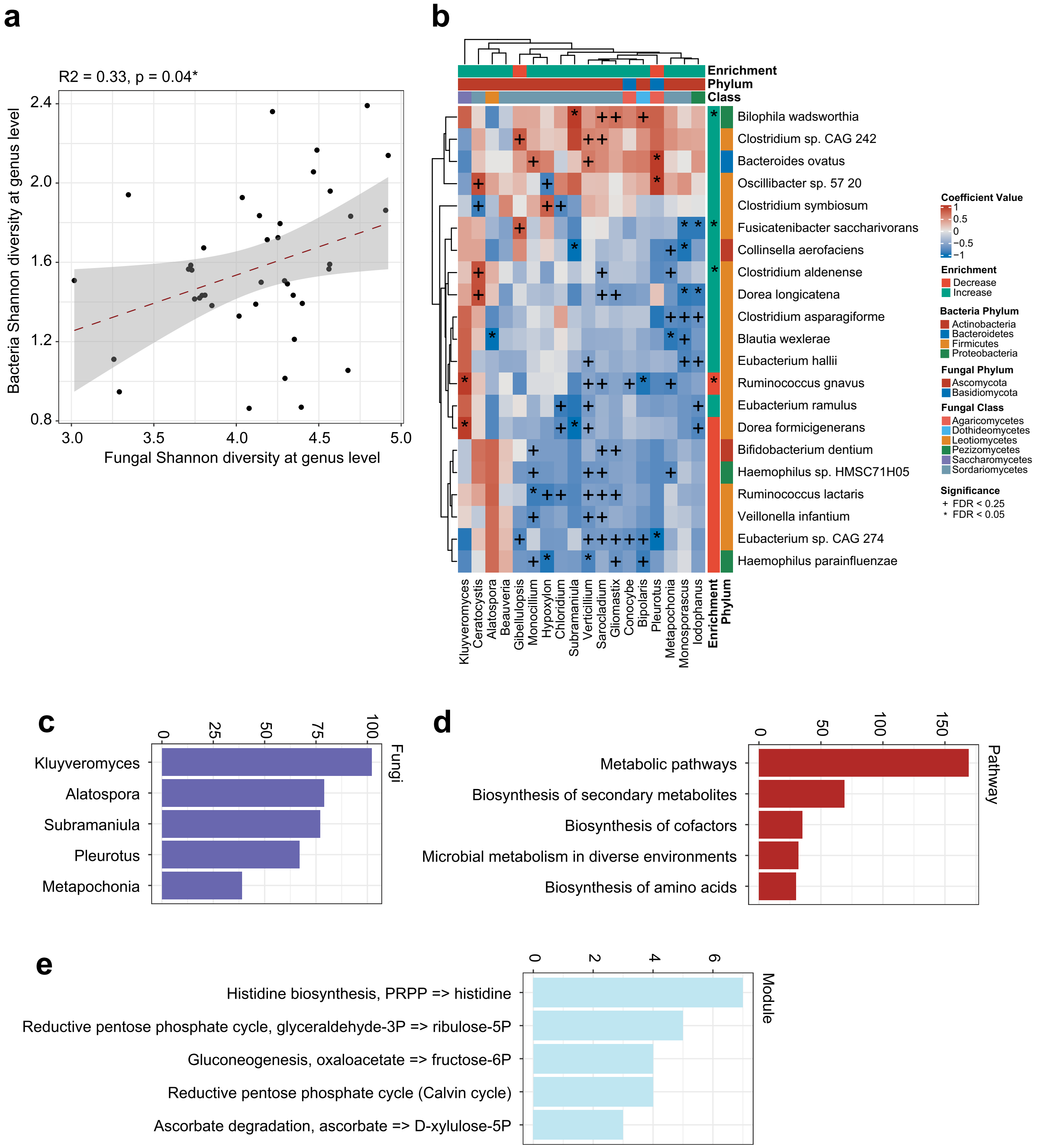

Figure 3. Inter-kingdom linkages between enteric fungi and bacteria in response to exercise intervention. (a) Significant correlation between gut fungal and bacterial α-diversity in Shannon indices at genus levels. Linear trends with a 95% confidence interval were shown. (b) Spearman correlation between the fold change of significant gut fungi and bacteria after exercise ($n = 20$). Significant correlations were marked with a plus symbol. The cells in red and blue represent positive and negative correlations. Significant correlations were marked with a plus symbol. *FDR <0.05, $^+$FDR <0.25. (c) The number of significant results of each fungus associated with bacterial KOs. The number of enteric fungi-related bacterial KOs categorized in the (d) KEGG pathway and (e) KEGG module.

analysis was performed within the circulating metabolites significantly correlated with the fungal community (Figure 4(a)). As shown, exercise-shaped fungi mainly affected amino acid metabolism pathways, including branched-chain amino acids (BCAA) biosynthesis, alanine, aspartate, and

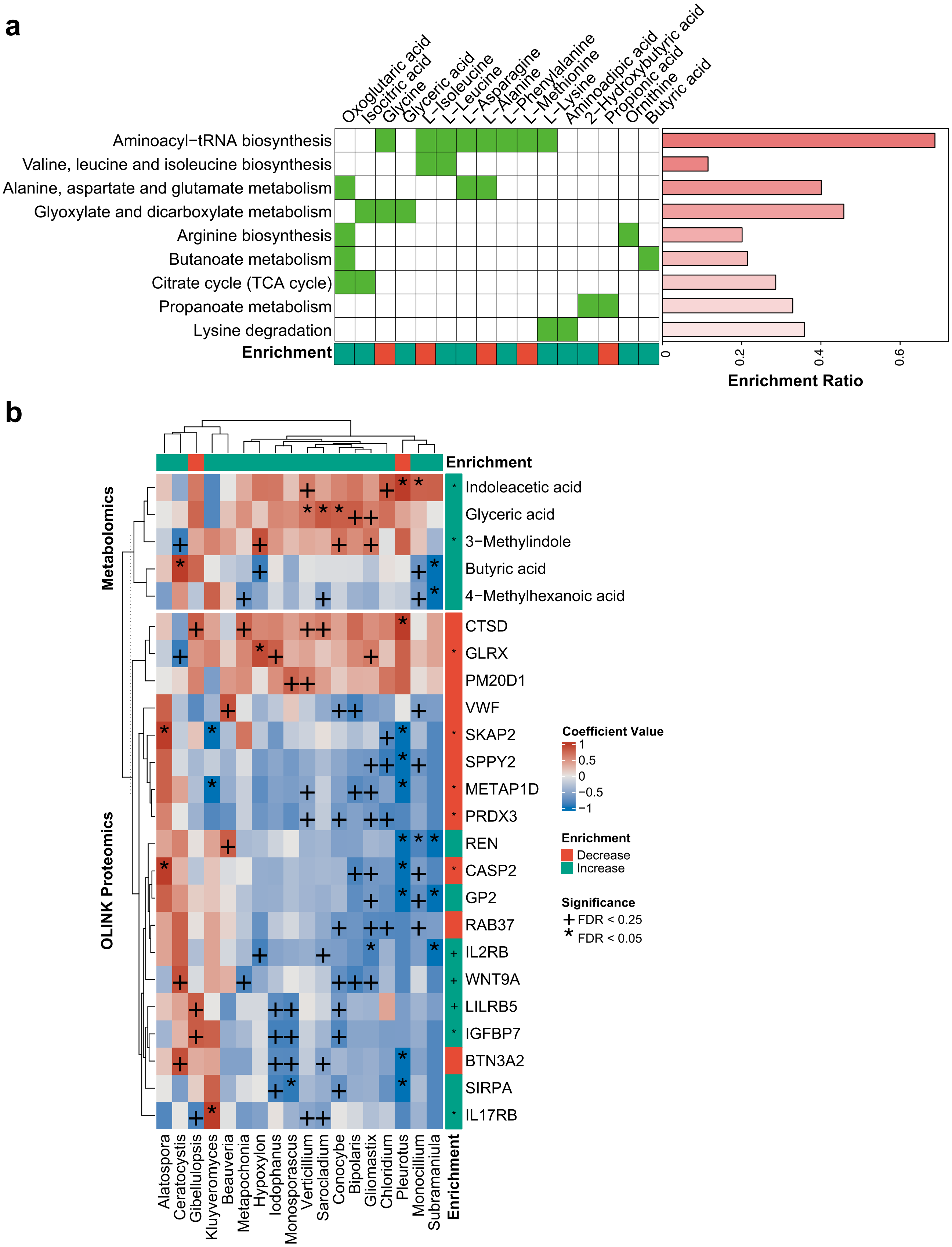

Figure 4. The correlations between changes in gut mycobiome and serum metabolome signatures, circulating proteomics profiles. (a) The pathway enrichment of metabolites which were significantly related to fungal changes. The cells marked in green indicated the involved metabolites in each pathway. The bar chart showed the enrichment ratio for each pathway and *P*-values from 0 to 0.05 were colored from red to white for each pathway. (b) The heatmap showed the Spearman correlation between the fold change in fungal abundance and serum metabolites and protein abundance ($n = 20$). The cells marked with plus symbols represent significant results. The cells in red and blue colors represent positive and negative correlations, respectively. *FDR <0.05, $^{+}$FDR <0.25.

glutamate metabolism, and arginine biosynthesis (FDR < 0.25). We further found exercise-promoted fungi associated with short-chain fatty acid (SCFA) metabolism with propanoate and butanoate enclosed. An intriguing observation was the positive association between butyric acid changes and *Ceratocystis* (FDR <0.05) (Figure 4(b), supplemental Figure S7). Considering the benefits of SCFA on host metabolism,[10] our data indicate that the growth of *Ceratocystis* may implicate in the benefits of exercise by promoting SCFA biosynthesis. Moreover, indoleacetic acid (3-IAA) is a microbial-derived tryptophan metabolite with anti-oxidation and autophagy effects.[52] We found that a significantly increased 3-IAA level was positively associated with exercise-altered *Verticillium* and *Chloridium* (FDR < 0.25) (Figure 4(b)). Altogether, we presented profound correlations between exercise-shaped gut fungal composition and alterations in circulating metabolites in serum.

Beyond metabolites, exercise also produces hormones and cytokines to systematically alleviate inflammation and restore metabolism.[19] We found that exercise-shaped gut mycobiome may affect host metabolism via regulating hormones and cytokines production (Figure 4(b) and Supplemental Figure S8). For instance, cathepsin D (CTSD) is a lysosomal protease upregulated in obesity,[53] its decline was aligned with the gain of *Gibellulopsis*, *Metapochonia*, *Verticillium*, and *Sarocladium* after training (FDR < 0.25). Moreover, the reduction trend of peptidase M20 domain containing 1 (PM20D1), a glucose metabolism-regulating enzyme increased in obese subjects and correlated with metabolic syndrome,[54] positively paralleled with increases in *Verticillium* and *Monosporascus* (FDR < 0.25). Similarly, exercise-increased *Gliomastix* and *Iodophanus* were positively aligned with reduced glutaredoxin-1 (GLRX) (FDR < 0.25), an activator of NF-κB signaling to accelerate adipogenesis.[55] Elevations in other metabolic syndromes-related factors, including serum insulin-like growth factor binding protein 7 (IGFBP7)[56] and leukocyte immunoglobulin-like receptor B5 (LILRB5),[57] were inversely accompanied by the proliferation of *Iodophanus*, *Monosporascus*, and *Conocybe*. In addition, interleukin 17 receptor B (IL-17RB) and IL-2 receptor B (IL2RB) are obesogenic genes in mice.[58,59] Their elevations were conversely related to higher *Sarocladium* abundance after exercise. By contrast, increased *Conocybe*, *Gliomastix*, and *Chloridium* were negatively linked to lower Ras-related protein 37 (RAB37, essential for glucose-stimulated insulin secretion[60] and Peroxiredoxin 3 (PRDX3, against glucose tolerance[61] (FDR < 0.25), signifying that changes in gut mycobiome might protect essential metabolic regulators from degradation. Glycoprotein 2 (GP2) is an intestinal M cells-sourced protein mediating gut inflammation.[62] It is also associated with exercise resistance in glycemic control.[19] Strikingly, the changing trend of GP2 was negatively associated with the gain of *Gliomastix* and *Monocillium*, suggesting that exercise-shifted gut fungi might be conducive to GP2 reduction. In conclusion, changes in the gut mycobiome are correlated with exercise-elicited metabolites and hormonal homeostasis status.

The potential impacts of baseline gut fungi on exercise outcomes revealed by integrative mediation analysis

With multi-omics approaches, we have elaborated strong associations between fungal alterations and exercise-induced benefits. To further clarify whether and how the initial mycobiome composition might determine the effectiveness of exercise, we conducted a mediation analysis to unearth the potential interplay between the baseline fungal abundance and the exercise-ameliorated host phenotypes.

The presence of *Ceratocystis* at baseline exhibited significant effects on exercise-reduced lipids levels including total cholesterol ($r = -1.1$, $p < 0.01$) and LDL (r=-0.91, $p < 0.01$) (Figure 5(a-c)). It was positively associated with linoleic acid ($r = 0.23$, $p < 0.01$, Figure 5(a)), which exhibits plasma cholesterol-lowering effects and is associated with a lower risk of heart disease in humans.[63] Interestingly, the initial *Ceratocystis* abundance was also inversely connected with PM20D1 (r=-0.38, $p < 0.01$, Figure 5(b, c)). This result was in line with our observation that changes in PM20D1 were parallel to the gain of *Verticillium* and *Monosporascus* post-training (Figure 4(b)), further underscoring that PM20D1 was a putative protein mediating gut mycobiome-host crosstalk.

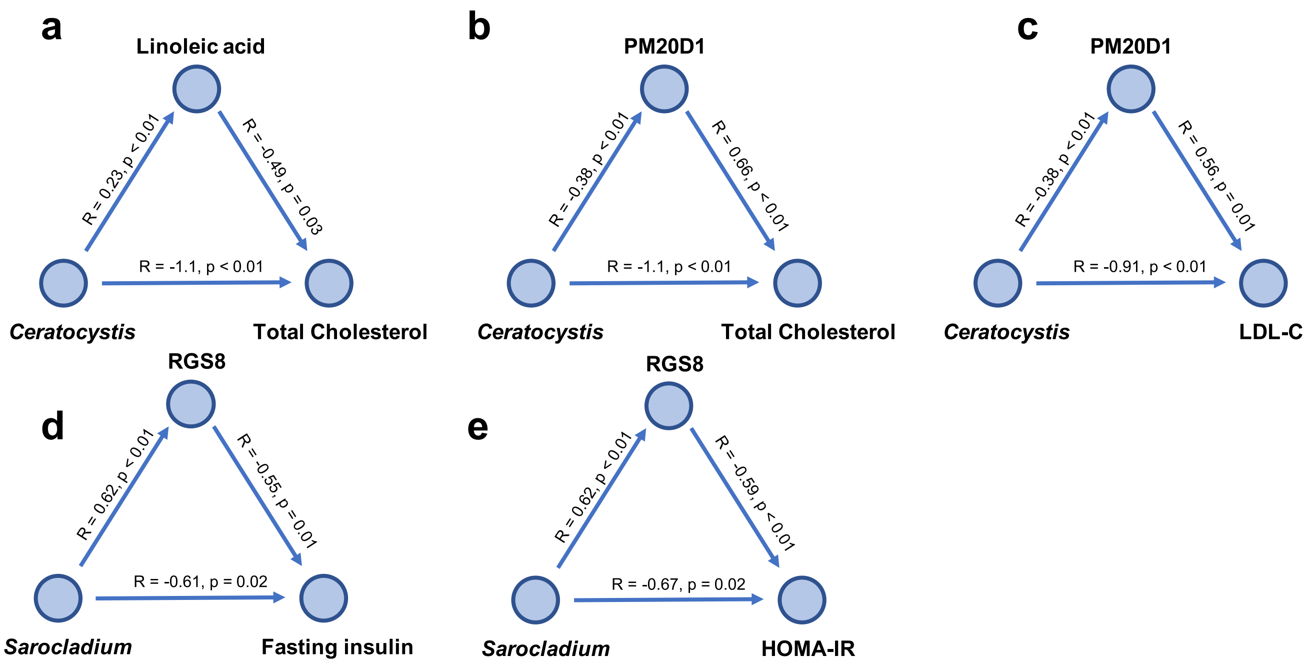

Figure 5. Basal gut fungal abundance is associated with exercise-improved outcomes via different mediators. The triangle plot showed the significant mediation effects from the fungal genus (left) to participants' phenotypes (right) via different metabolites, and serum proteins (middle). The arrows showed the direction of the effect and the corresponding Spearman coefficient and p values were indicated.

Sarocladium emerged as another fungus of interest, with potential benefits for host insulin sensitivity regarding fasting insulin ($r = -0.61$, $p = 0.02$) and HOMA-IR ($r = -0.67$, $p = 0.02$) (Figure 5(d, e)). This data was supported by our prior finding that alternations in *Sarocladium* were associated with improvements in insulin sensitivity (Figure 2(a)). Our results indicated the insulin-sensitizing effects of *Sarocladium* potentially via modulating Regulators of G-protein Signaling 8 (RGS8), key downstream elements of G protein-coupled receptors (GPCRs) regulating insulin secretion and glucose homeostasis.[64] Of note, besides their impact at baseline, *Ceratocystis* and *Sarocladium* significantly increased post-exercise by 30.0% and 50.7%, respectively (Figure 1(f)), which further potentiated the importance of gut mycobiome in exercise-induced benefits.

Applying baseline gut mycobiome features predicts exercise responsiveness in terms of insulin sensitivity

We have proved that gut bacterial microbiome profiles determine personal exercise outcomes concerning insulin sensitivity and glycemic control.[10] Hereby, we subsequently interrogate whether alternations in the gut mycobiome are associated with exercise responsiveness. Exercise responders (R) and non-responders (NR) were identified based on their improvement in homeostasis model assessment of insulin resistance (HOMA-IR) after training as published.[10] We identified 36 R and 14 NR when pooling the discovery and validation cohort together. Though overall fungal diversity between R and NR was comparable (Supplementary Figure S9), a distinct pattern in the fungi components was observed in the two subgroups. *Hanseniaspora* is associated with hyperglycemia in women with gestational diabetes,[65] which was exclusively increased in NR (Figure 6(a)). Conversely, *Cystobasidium*, a predominated fungus in euglycemic subjects,[66] decreased significantly in NR after exercise training. In R, elevations in *Paraphoma* and *Debaryomyces*, two fungal genera enriched in healthy controls than in diabetes patients,[66,67] were exclusively observed. Of note, alternations in *Paraphoma* were positively associated with changes in serum levels of histidine (Supplementary Figure S10), which corroborated

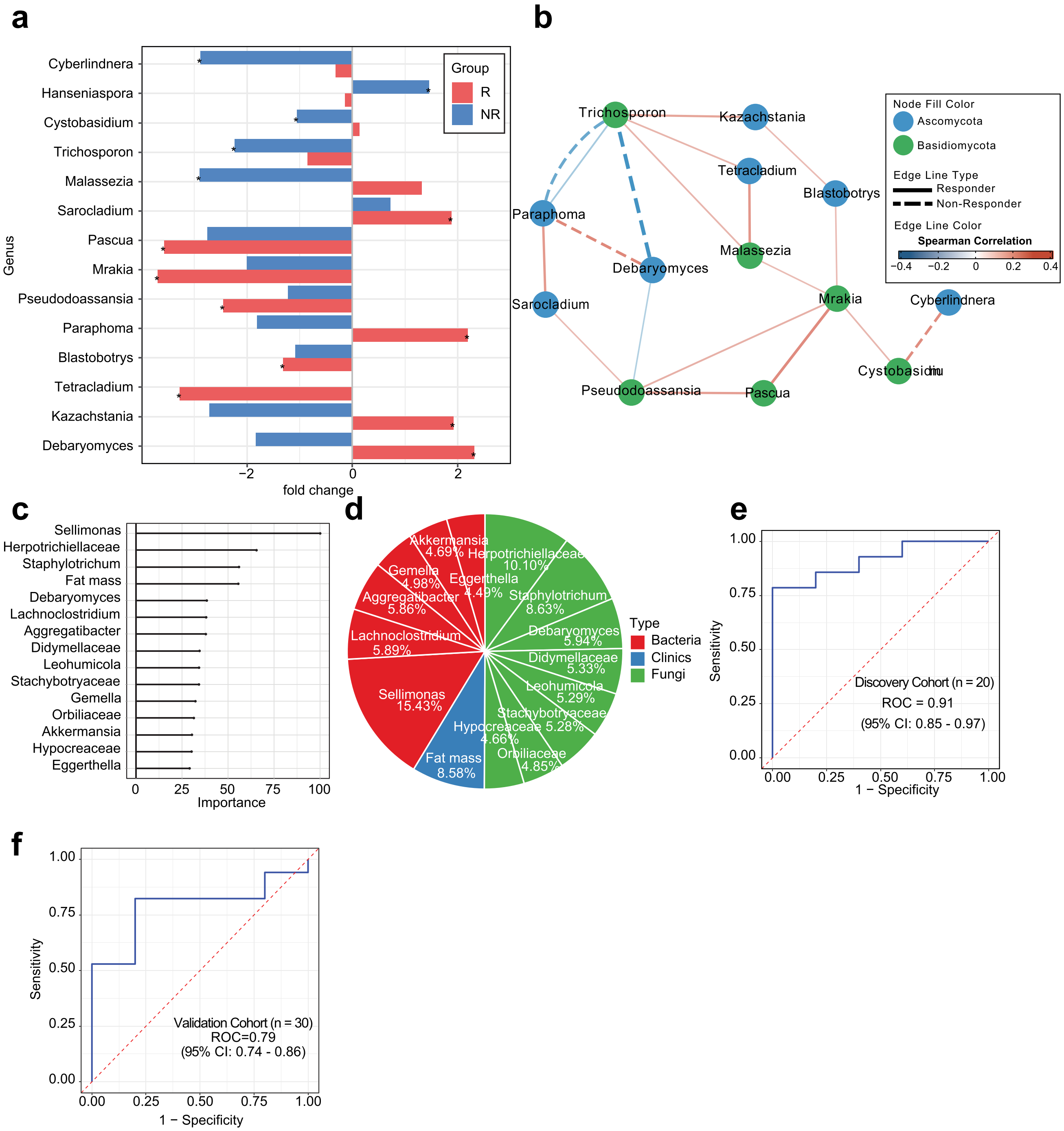

Figure 6. Baseline gut mycobiome predicts exercise responsiveness in the improvement of insulin sensitivity. (a) The significantly changed fungal genera between exercise responders (R, $n = 36$) and non-responders (NR, $n = 14$) after training. (b) The correlation networks among the fold changes in significantly changed genera in R and NR were constructed. Genera were colored according to their affiliated phyla. The correlations in R and NR were connected in solid and dashed edges, respectively. The edges colored from blue to red represented the coefficient value from – 1 to 1 for the significant correlations. (c) The informative feature plots show the importance of the selected mycobiome in the machine-learning model.(d) the pie chart shows the percentage of the importance of informative features used in the prediction model. The pie colors indicate the different catalogs, including fungi, bacteria, and clinical information. (e) The receiver operating characteristic curves and area under the curve (AUROC) of the mycobiome-based predictive models for discriminating R and NR in the discovery cohort ($n = 20$). (f) AUROC of mycobiome-based predictive models to identify NR in the validation cohort ($n = 30$).

our findings that histidine biosynthesis was the most enriched pathway after exercise training. Moreover, the enrichment of *Kazachstania* in R was supported by previous findings that *Kazachstania* was associated with normal body weight and circulating HDL level.[68] The intra-kingdom linkages at the genus level in R and NR were 14 and 4, respectively (Figure 6(b)), suggesting R exhibited a more active crosstalk within the fungal ecosystem than NR.

The gut microbial fingerprint is useful for predicting the outcomes of various interventions, including diet, exercise, and medication.[10,69,70] We therefore investigated the predictive value of fungal signatures on individuals' exercise responsiveness. Eight fungal genera, 6 bacteria genera, and fat mass at baseline were selected in the prediction models (Figure 6c and Supplemental Table-S8), where fungal information contributed over 50% importance to this algorithm (Figure 6(d)). Among informative features, *Herpotrichiellaceae* and *Didymellaceae* were enriched in NR, while *Hypocreaceae* and *Stachybotryaceae* were enriched in R (Supplementary Figure S11). Using this model with machine learning methods, we achieved an area under the receiver operating characteristic (AUROC) of 0.91 (95% CI: 0.85 to 0.97) to discriminate the R and NR within our discovery cohort ($n = 20$, Figure 6(e)). Further evaluation in the validation cohort ($n = 30$) also resulted in an AUROC of 0.79 (95% CI: 0.74 to 0.86) (Figure 6(e)). Remarkably, this combined model significantly outperformed the validation cohort compared to algorithms based solely on either fungal or bacterial features with clinical data ($p < 0.05$) (Supplemental Figure S12), suggesting a potential synergistic interaction between gut-resident bacteria and fungi in determining exercise responsiveness. Therefore, our findings further emphasized the importance of targeting gut fungi to develop a personalized exercise regimen in diabetes prevention.

Discussion

In this clinical study, we uncover that exercise significantly shapes gut mycobiome composition, which extends our current understanding of exercise impacts beyond gut-resident bacteria to fungi. The dynamics of enteric fungi are closely associated with exercise-induced changes in the bacterial microbiome, metabolome, and proteome, indicating that commensal fungi might be a possible contributor to exercise-induced metabolic benefits. Furthermore, we observed differential changes in the gut mycobiome between exercise responders and non-responders. We subsequently developed a machine-learning algorithm integrating baseline gut mycobiome profiles to predict exercise responsiveness in glycemic control and insulin sensitivity. Our results highlight the potential to precisely tailor exercise by targeting gut fungal profiles.

The importance of the bacterial microbiome in exercise has been elucidated, whereas the contribution of gut fungi remains under-investigated. We demonstrated that exercise leads to effective improvements in gut fungal diversity and intra-kingdom communication as evidenced by higher α-diversity and enriched fungal co-abundance network. These findings corroborate previous works that both dietary intervention and Roux-en-Y gastric bypass surgery remarkably remodel the gut mycobiome taxonomy.[13,71] Remarkably, unlike the bacterial microbiome, which retains compositional stability responding to exercise,[10] our data indicates high plasticity and adaptability of the gut mycobiome to lifestyle modification. Nevertheless, there is still an absence of studies to directly compare the changes in these two microbial communities in response to an identical intervention. The stability and resilience of gut bacteria and fungi may vary across different conditions and warrant further investigation. Interestingly, we observe a linear correlation between fungal and bacterial diversity after training, indicating an interdependent response of commensal fungi and bacteria to exercise. Like intestine bacteria, which implicates exercise-promoted benefits through regulating microbial metabolites,[10] the changes in fungal composition are also associated with SCFA and amino acid metabolism. These results collectively imply a synergic and coexisting interaction between gut-colonized bacteria and fungi during exercise. Given those extensive linkages we observed, our results further suggest that, apart from commensal bacteria, gut fungi also serve as a potential molecular transducer for physical exercise.

We further identified a subset of fungi that significantly increased after exercise training. For instance, *Verticillium* shows the most pronounced response to exercise with a 1.5-fold augment. Its elevation correlates closely with the exercise-induced improvement in fasting glucose level, body composition, and strength performance. Our integrated omics analysis further suggests that *Verticillium* may exert benefits through multiple pathways. A noteworthy microbial interaction is between changes in *Verticillium* with *Veillonella infantium*, a lactic acid-producing bacterium associated with exercise performance,[43] which indicated that growth in *Verticillium* might foster a conducive environment for the proliferation of bacterial strains with metabolic benefits. 3-IAA is a microbial metabolite that determines chemotherapy responsiveness in pancreatic cancer.[52] The positive association between increased circulating 3-IAA levels and exercise-induced fungi genus raised the possibility that IAA may serve as a transducer mediating Verticillium-derived benefits, which warrants further investigation in the future study. Besides, change in *Verticillium* may also contribute to PM20D1's reduction, a known biomarker for human metabolic syndrome.[54] In addition, abundance of *Sarocladium* was also 50% higher after exercise training and associated amelioration in insulin resistance. Our results also suggested *Sarocladium* as a potential regulator of host glucose metabolism by facilitating the gain of anti-obese gut bacteria, including *V. infantium* and *Bifidobacterium dentium*. In concordance that *Ceratocystis* level is inversely associated with glucose tolerance and insulin resistance,[13] we found 30% increases in *Ceratocystis* in response to exercise intervention. Importantly, our mediation analysis indicates that the baseline abundance of *Ceratocystis* and *Sarocladium* may influence the outcome of exercise by modulation of metabolites and cytokines levels. However, whether these fungi are symbiotic residents or transient environmental species remains debatable.[72] Nevertheless, our results revealed the potential links between exercise-altered fungi, gut bacteria, and serum immune factors. Further investigation into those exercise-shifted fungal genera through culturomics and mechanistic elucidation is needed to establish their capacity for colonization and adaptation within the human gastrointestinal tract and their specific contributions to immune regulation, which are vital features to distinguish the true symbiotic.[72] Taken together, our integrative omics analysis uncovers the possible linkages between gut mycobiome and host metabolism from multiple dimensions.

Exercise resistance poses a major challenge in its clinical application for diabetes prevention and management.[10,73] The difference in the gut bacterial microbiome has been implicated as a potential determinant of individual responsiveness to various interventions.[10,69,70] A high abundance of commensal fungi *Candida* in human recipients is correlated with the therapeutic efficacy of fecal microbiome transplantation for colitis.[74] Herein, we observed distinct changes in the gut mycobiome composition between exercise responders and non-responders. Notably, a machine-learning algorithm mainly based on basal gut fungal signatures accurately predicts exercise responsiveness in improvements of glycemic control and insulin sensitivity with an AUC of 0.91, which also achieved an AUC of 0.79 in our validation cohort. Importantly, the fungal information contributes more than 50% importance in this model, suggesting that the gut mycobiome may serve as potential biomarkers for monitoring and predicting exercise outcomes. The predictive power of this new model is comparable with our previous model established by using features combining 15 circulating metabolites and 13 gut bacterial species,[10] which requires both sophisticated liquid chromatography-mass spectrometry (LCMS)-based blood measurement of metabolites and fecal shotgun sequencing. On the other hand, our current algorithm is based only on fat mass and sequencing data from stool samples, which is relatively easier to implement and is more cost-effective than the previous prediction model. Although further validation and optimization of this fungi-based prediction model is required in large, independent cohorts and clinical implementation remains distant, our findings provide advanced evidence for the close association between gut fungal composition and exercise responsiveness with respect to insulin sensitivity and glucose metabolism.

Our study has several limitations that warrant further discussion. First, due to the technical limitation of ITS2 sequencing, we only decipher the fungal taxonomy at genus levels. Further exploration of fungal species information and function annotations is warranted using advanced sequencing techniques. Second, our association analysis only unveils conceivable mechanistic linkages between gut mycobiome and exercise-mediated metabolic improvement. The explore and correlational nature of our data precludes the establishment of causal relationships. Further mechanistic experiments are needed to establish their cause–effect relationship and the underlying biological pathway whereby altered fungal species modulate host metabolism. Ideally, mono-colonization of fungal species responsive to exercise intervention is required to elucidate their specific metabolic roles *in vivo*. However, the current technology for identifying, isolating, and culturing single fungal species is rather limited, thereby hindering further detailed mechanistic studies. Additionally, our study is also limited by the small sample size, due to logistical difficulties in recruiting a larger number of eligible medication-naïve overweight individuals with prediabetes for performing supervised high-intensity exercise studies. As a result, the conclusions drawn from this study should be interpreted with caution. Another limitation is that our study population includes Chinese participants only. It remains unclear whether our prediction model for exercise responsiveness can be generalized in different populations. Further studies with larger sample sizes and diverse ethnic groups are needed to consolidate our findings on the changes in gut mycobiome in response to exercise training.

Conclusion

In summary, this integrative multi-omics study elucidates that exercise profoundly shapes gut mycobiome composition, with its dynamics tightly associated with exercise-induced metabolic benefits. These findings suggest that gut mycobiome might be an important regulator of host metabolism and raise the possibility of targeting gut mycobiome as a novel approach for personalized lifestyle intervention for preventing diabetes and other metabolic disorders. While our results shed new light on the possible involvement of the gut mycobiome in exercise-shaped metabolism and insulin responsiveness, further larger-scale studies in independent cohorts and mechanistic investigations are warranted to validate our findings and to dissect the cause–effect relationship between gut myobiome and exercise physiology.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Authors’ contribution

Y. W. and J. C. conceived and designed the study, performed the data analysis, interpreted the results, and drafted the manuscript; Y. N. and Y. L. assisted in the data analysis and discussion; X.G. helped to extract fecal DNA. M.A.T. revised the manuscript; G.P. supervised the bioinformatics analysis and edited the manuscript. A.X. supervised the whole study and wrote and edited the manuscript. All authors read and approved the final manuscript.

Data availability statement

Metagenomic sequencing data for all samples have been deposited in NCBI Sequencing Read Archive under BioProject ID: PRJNA454826. ITS2 sequencing data has been deposited in NCBI Sequencing Read Archive under BioProject ID: PRJNA1011537. Other data sets of this study are available upon reasonable request.

Ethics approval

The study was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (UW15–370) and registered on ClinicalTrials.gov (NCT0324097). Written informed consent was obtained from each patient before enrollment.

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